UNIVERSITY OF ALBERTA

ROLE OF IL-15 TRANSPRESENTATION IN MEMORY CD8$^+$ T-CELL RESPONSES

by

©

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ABSTRACT

$CD8^+$ T-cells are a population of lymphocytes that mediate cytotoxic activity against transformed and virally infected cells. IL-15 is a pleiotropic cytokine involved in diverse aspects of $CD8^+$ T-cell function. Among cytokines, IL-15 is unique since it is coordinately expressed on the cell surface with the IL-15Rα chain and presented in *trans* to opposing cells. Using cell-sized microspheres, the specific contribution of transpresented IL-15 to $CD8^+$ T-cell binding, homeostasis and reactivation was examined.

Transpresented IL-15 has a rapid and profound effect on the binding and aggregation of $CD8^+$ T-cells, particularily the central memory (T_{CM}) subset. Live-cell imaging highlighted the adhesive properties of transpresented IL-15, which resembled "tethered" adhesion mediated by surface-bound chemokines. Functionally, transpresented IL-15 in the absence of any additional signals could maintain the long-term survival and antigen-specificity of lymphocytic choriomeningitis virus specific $CD8^+$ T-cells *in vitro*. The capacity of IL-15 to reactivate memory $CD8^+$ T-cells was next examined. It was found that transpresented IL-15 is required for optimal recall responses by memory $CD8^+$ T-cells. In pursuing this further, T_{CM} $CD8^+$ T-cells were found to be more responsive to transpresented IL-15 than effector memory (T_{EM}) $CD8^+$ T-cells both *in vitro* and *in vivo*. Therefore, in addition to its well known role as a pro-survival cytokine, transpresented IL-15 functions as an adhesion molecule and as a memory $CD8^+$ T-cell specific costimulatory ligand.

The lack of defined phenotypic markers for memory $CD8^+$ T-cells remains a major obstacle. Currently, no single marker can be used to identify memory $CD8^+$ T-cells since many memory markers are commonly expressed by lymphocyte populations in various stages of differentiation. For this reason, the reactivity of a novel anti-Ly-6C mAb (iMap) developed in our laboratory was characterized. It was found that only high level expression of Ly-6C on $CD8^+$ T-cells correlated with a functional memory phenotype.

Importantly, iMap did not react with Ly-6C expressed on resting CD4$^+$ T-cells as detected by another anti-Ly-6C mAb AL-21. Due to its restricted expression pattern, high iMap reactivity could be used a single marker to identify and isolate functional memory CD8$^+$ T-cells from the secondary lymphoid organs.

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ABBREVIATIONS

2C11Rα	Microspheres bearing immobilized anti-CD3ε (clone: 145-2C11) + IL-15Rα/Fc
2C11Rα15	Microspheres bearing immobilized anti-CDε (clone: 145-2C11) + IL-15Rα/Fc:IL-15
2-ME	β-2-mercaptoethanol
A-LAKs	Adherent lymphokine-activated killer cells
APC	Antigen presenting cell
aAPC	Artificial antigen presenting cell
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BM	Bone marrow
BMDC	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
CANVAC	Canadian Networks for Vaccines and Immunotherapeutics
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFDA-SE	5- (and 6-)carboxyfluorescein diacetate succinimidyl ester
CFSE	5- (and 6-)carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T-lymphocyte

DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
E:T	Effector to target
ELISPOT	Enzyme linked immunospot
F(ab')$_2$	Dimer of an antigen binding fragment
FACS	Fluorescence activated-cell sorter
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
g	Gram
GM-CSF	Granulocyte macrophage colony stimulating factor
gp	Glycoprotein
gp33/Rα	Microspheres bearing immobilized gp33 peptide pulsed H-2D^b/Ig + IL-15Rα/Fc
gp33/Rα15	Microspheres bearing immobilized gp33 peptide pulsed H-2D^b/Ig + IL-15Rα/Fc:IL-15
GPI	Glycophosphatidyl-inositol
grB	Granzyme B
H-2	Mouse major histocompatibility complex
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid
hr	Hour
HRP	Horseradish peroxidase

ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-15Rα	IL-15 receptor alpha
iMap	Immunologic memory associated protein
i.p.	Intraperitoneal
i.v.	Intravenous

K_d	Dissociation constant
kDa	Kilodalton

L	Litre
LCMV	Lymphocytic choriomeningitis virus
LFA	Leukocyte (lymphocyte) function-associated Ag
LN	Lymph node
LPS	Lipopolysaccharide
$\text{Ly-6C}^{\text{AL-21}}$	Ly-6C identified by the AL-21 mAb
$\text{Ly-6C}^{\text{iMap}}$	Ly-6C identified by the iMap mAb

μ	Micro-
m	Milli-
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minute
MP	Memory phenotype

MPEC	Memory precursor effector cell
mRNA	messenger ribonucleic acid
n	Nano-
NK	Natural killer
NP	Nucleoprotein
NP366/Rα	Microspheres bearing immobilized NP366 peptide pulsed H-2D^{b}/Ig + IL-15Rα/Fc
NP366/Rα15	Microspheres bearing immobilized NP366 peptide pulsed H-2D^{b}/Ig + IL-15Rα/Fc:IL-15
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PFHMII	Protein free hybridoma media II
PFU	Plaque forming unit
pMHC	Peptide major histocompatibility complex
poly I:C	Polyinosinic:polycytidlic acid
R	Receptor, (e.g. IL-15R)
Rα	Microspheres bearing immobilized IL-15Rα/Fc
Rα15	Microspheres bearing immobilized IL-15Rα/Fc:IL-15
R-PE	Recombinant phycoerythrin
RPMI-1640	Roswell Park Memorial Institute formulation 1640
SEM	Standard error of the mean
SLEC	Short lived effector cell

T_{CM}	Central memory
TCR	T-cell receptor
T_{EM}	Effector memory
TLR	Toll-like receptor
TNF	Tumor necrosis factor
w/v	Weight to volume ratio

CHAPTER 1

GENERAL INTRODUCTION

During life, there is a never ending battle against a vast array of infectious pathogens. To survive these challenges, the immune system has evolved the ability to recognize and neutralize an ever changing array of foreign microorganisms. For the most part, the body's many innate defense systems provide a formidable first line of defense (1). Occasionally however, innate defenses are overwhelmed by pathogens that have evolved strategies to subvert or avoid elimination by the innate immune system (2). Such infections require more elaborate and flexible defense strategies mediated by the adaptive immune system. Various lymphocytes subsets make up the adaptive immune system and each plays an important role in quelling infection. Natural killer (NK) cells, NK-T cells, and $\gamma\delta$ T-cells are innate immune lymphocytes that serve as a first line of defense against invading pathogens in peripheral organs and tissue. T-cells and B-cells are the two major lymphocyte populations of the adaptive immune system. B-cells are primarily responsible for humoral immune responses whereas, T-cells are involved in cell-mediated immunity. For the most part, $CD4^+$ T-cells produce cytokines that help direct the immune response, while $CD8^+$ T-cells are responsible for killing infected and transformed cells. Within the last 30 years, significant progress has been made in understanding how $CD8^+$ T-cells recognize, expand in number, and provide memory to previously encountered pathogens to offer lifelong protection against reinfection (3). The focus of my thesis work has been on the study of mouse $CD8^+$ T-cells as an approachable model to provide insights into understanding human $CD8^+$ T-cell responses.

Historically, immunology developed from attempts to understand the infectious disease pathogenesis and prevention (4). Due to the enormous progress in biochemistry, molecular biology, embryology, and animal physiology, many of the ground rules underlying immune responses have been identified. With regards to understanding CD8$^+$ T-cell biology, studies conducted in mice with well-characterized mouse pathogens such as lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* have provided the bulk of recent advances (5). Although it is only one example, a major technological breakthrough came when John Altman, Mark Davis, and their colleagues, developed peptide antigen major histocompatibility complex (MHC) class I tetramers (6). These reagents revolutionized tracking and monitoring of antigen-specific CD8$^+$ T-cell responses, at the single cell level. Around the same time, enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS) techniques were developed that allowed the functional examination of antigen-specific CD8$^+$ T-cell responses, also at the single cell level (7, 8). The contribution of these three techniques cannot be overstated, since they allowed the phenotypic and functional assessment of antigen-specific CD8$^+$ T-cells directly *ex vivo*, without the need for *in vitro* restimulation. Fluroescent labeling of CD8$^+$ T-cells with 5- (and 6-) carboxyfluorescein succinimidyl ester (CFSE) provided a means determine both cell division and cellular localization *in vivo* (9). T-cell receptor (TCR) transgenic mice combined with adoptive transfer techniques provided a method to visualize the early events in CD8$^+$ T-cell activation and differentiation *in vivo* that would have otherwise been too low in frequency to be detectable (10, 11). Together, these technologies were used to demonstrate that the majority of CD8$^+$ T-cells that expand during acute infection are in fact, pathogen specific (12). More importantly, research stemming from the use of these techniques has provided a greater appreciation for the phenotypic and functional complexity among CD8$^+$ T-cells.

CD8$^+$ T-CELL RESPONSE TO ACUTE INFECTION

A typical CD8$^+$ T-cell response to acute infection consists of three characteristic phases: (*i*) during the priming phase, naive CD8$^+$ T-cells undergo massive clonal proliferation, acquire effector function, migrate to sites of infection, and mediate pathogen clearance; (*ii*) during the contraction phase, the majority of the activated effector CD8$^+$ T-cells die by apoptosis, leaving behind a small surviving fraction that persists as long-lived memory cells; and (*iii*) during the memory maintenance phase, memory CD8$^+$ T-cells are maintained at numerically stable levels for essentially the life of the mouse ((13, 14), Fig. 1-1). The resulting memory CD8$^+$ T-cells are endowed with unique properties that permit more vigorous responses following secondary exposure to antigen, thereby offering enhanced protection to the host (14-17).

PRIMARY CD8$^+$ T-CELL ACTIVATION

CD8$^+$ T-cells recognize specific pathogen-derived peptides presented by self MHC class I molecules via their clonotypic TCR (18). To ensure the capacity to respond to the enormous diversity of potential pathogens, the number of naïve CD8$^+$ T-cells specific for any given antigenic peptide is extremely low, and has been estimated to be in the range of ten to several hundred per laboratory mouse (19-25). Naïve CD8$^+$ T-cells do not have immediate effector mechanisms, nor do they have the ability to enter peripheral tissues to combat infections (26). Rather, naïve CD8$^+$ T-cells circulate between the blood and secondary lymphoid organs by virtue of specific homing and chemokine receptors (15, 27). It is within these secondary lymphoid organs that naïve CD8$^+$ T-cells receive signals necessary for their activation from specialized antigen presenting cells (APC), such as dendritic cells (DC) (28, 29). Dendritic cells are present throughout peripheral tissues and organs, continuously ingesting antigen and presenting processed antigenic peptides in the context of MHC on their cell surface (28, 29). Under normal conditions, presentation of peptide/MHC complexes (pMHC) by immature DCs is limited to self or environmental peptides. As a consequence of infection, pathogen products engage toll-like receptors (TLRs) expressed by DCs, resulting in their activation. This leads to increased cytokine secretion and cell surface expression of pMHC and costimulatory molecules. Activation of DCs is also associated with their migration to secondary lymphoid organs. In this context, mature antigen-laden DCs can initiate the full scale activation of naïve CD8$^+$ T-cells (28-31).

Direct visualization of CD8$^+$ T-cell – DC conjugates in intact lymph nodes has recently demonstrated that this interaction can be divided into three phases (32). The first phase is characterized by brief CD8$^+$ T cell – DC interactions that occur during the first 8 hours following antigen encounter. These initial interactions are accompanied by the upregulation of early activation markers by the antigen-specific CD8$^+$ T-cells. The second phase occurs in the subsequent 12 hours, during which CD8$^+$ T-cells undergo slower migration and maintain prolonged contacts with DCs. By 48 hours after initial antigen encounter, these tight conjugates break apart, and CD8$^+$ T-cell expansion and differentiation occurs with only minimal DC contact.

During the interaction with mature DCs, three major classes of signals are provided to the naïve CD8$^+$ T-cells to initiate their activation program. Signal 1 is delivered via pMHC/TCR interactions, which provides antigenic specificity to the response. Signal 2 is delivered by a variety of costimulatory molecules such as B7-1 and -2 that are expressed by mature DCs (33-35). Signal 3 is provided by type I interferon (IFN) and interleukin-12 (IL-12) which restrict responses so that they only occur in the presence of inflammation (36). By requiring multiple signals for full activation, CD8$^+$ T-cell responses can be regulated to minimize the risk of developing immune cell mediated pathology and autoimmune disease (37).

Following activation, CD8$^+$ T-cells embark on a program of proliferation and differentiation. In as few as eight days following infection, activated CD8$^+$ T-cells can expand as much as 10^4- to 10^5-fold, with each cell undergoing 10-20 divisions (11, 12, 38). Moreover, this proliferation is accompanied by extensive cellular differentiation into effector CD8$^+$ T-cells that leave the secondary lymphoid organs and disseminate through the bloodstream into peripheral tissues (39-41). Entry of activated CD8$^+$ T-cells into peripheral tissue is largely attributable to changes in the expression of various chemokine receptors and cell adhesion molecules (26). Upon arrival in inflamed tissues, effector CD8$^+$ T cells control infection through direct cytotoxic activity (via perforin and granzymes) and the secretion of in IFN-γ and tumor necrosis factor α (TNF-α) (14, 42-44). Once the infection has been resolved, the majority of the effector CD8$^+$ T-cells die by apoptosis in a process known as contraction (15, 45). In general, contraction eliminates 90–95% of pathogen-specific effector CD8$^+$ T cells, presumably to preserve immune system homeostasis, thereby preventing CD8$^+$ T cells specific for a single pathogen from indefinitely dominating the immune repertoire. Importantly, although contraction is substantial, it is incomplete and some pathogen-specific CD8$^+$ T-cells survive (39, 46). The surviving few, consist of antigen-experienced CD8$^+$ T-cells that over time progressively develop into long-lived memory cells.

CD8$^+$ T-CELL MEMORY

The ability to develop immunologic memory to previously encountered antigens is a defining hallmark of the adaptive immune system. On a per cell basis, memory CD8$^+$ T-cells provide better protection than naïve CD8$^+$ T-cells against the same pathogen. This enhanced protection is due to: (*i*) increased numbers of antigen specific CD8$^+$ T-cells in the memory repertoire; (*ii*) their ability to persist for extended periods of time due to antigen-independent homeostatic proliferation; and (*iii*) their capacity to rapidly acquire effector function and undergo vigorous secondary expansion upon re-infection (15-17, 47-49)

CELL SURFACE MARKERS OF MEMORY CD8$^+$ T-CELLS

Various cell surface markers can be used to distinguish naïve, effector and memory CD8$^+$ T-cell populations; however, there is considerable heterogeneity in terms of the expression of these phenotypic markers (15, 47, 49). Although it has been highly sought after, no single marker has been found that can be used to unequivocally identify a single CD8$^+$ T-cell population. Another major complication is that during the transition from a naïve to a memory phenotype, many cell surface markers are commonly expressed by various lymphocytes and their respective subpopulations (50, 51). CD44 is a widely used memory marker that is also highly expressed by effector CD8$^+$ T-cells (52, 53). CD62L and the chemokine receptor CCR7 are also dynamically expressed during the course of an immune response (47). Both CD62L and CCR7 are found on naïve CD8$^+$ T-cells, downregulated during the effector stage and subsequently re-expressed by a subpopulation of memory CD8$^+$ T-cells. Other functional and phenotypic markers expressed at various stages of development include adhesion ligands, chemokine and cytokine receptors, and ligands of unknown function (Table 1-1). Ly-6C is an example of a memory marker with unknown function (54). Ly-6C is a small glycophosphatidyl inositol (GPI)-anchored membrane protein whose expression has been correlated with a memory phenotype, although it does not appear to be required for the normal function or development of CD8$^+$ T-cells (55, 56). Clearly, CD8$^+$ T-cells modulate the expression of several phenotypic markers as they progress to the memory stage; therefore, multiple criteria are typically used to identify CD8$^+$ T-cell populations.

MEMORY CD8$^+$ T-CELL HETEROGENEITY

Based on phenotypic markers, anatomical location, and function, memory CD8$^+$ T-cells can be broadly divided into two distinct subpopulations (39, 40). These designations, originally derived from analysis of human memory T-cells, describe central memory (T_{CM}) and effector memory (T_{EM}) T-cells. By virtue of the expression of the lymph node homing molecules CD62L and CCR7, T_{CM} CD8$^+$ T-cells preferentially reside in secondary lymphoid organs such as the spleen and lymph nodes. Conversely, T_{EM} CD8$^+$ T-cells lack expression of both CD62L and CCR7 and are predominantly found in non-lymphoid peripheral tissues such as the lung, liver and intestine, but can also be found in the spleen (49, 57). From a functional perspective, initial studies suggested that T_{EM} CD8$^+$ T-cells acquire effector functions, such as cytokine production and killing more rapidly than T_{CM} CD8$^+$ T-cells (39, 40, 58). However, findings that are more recent suggest that upon antigen re-challenge, both subsets are nearly equivalent in their production of effector cytokines and cytotoxicity (49, 59). When compared on a per cell basis, T_{CM} CD8$^+$ T-cells confer better protective immunity from re-infection than T_{EM} CD8$^+$ T-cells due to their enhanced proliferative capacity (59-63). Although the division of memory CD8$^+$ T-cells into these two subpopulations may be overly simplistic, the concept of T_{EM} and T_{CM} has remained an important paradigm in the memory field.

MEMORY CD8$^+$ T-CELL DEVELOPMENT

The development and lineage relationship between the various CD8$^+$ T-cell populations during an immune response is a controversial topic, and continues to be a subject of ongoing debate (13, 14, 45, 64-68). As such, numerous models have been proposed to account for the heterogeneity seen in the CD8$^+$ T-cell population. Key discoveries in the last few years have shed new light on these topics and have shaped the current understanding of these processes. In 2003, Wherry *et al.* first proposed that distinct CD8$^+$ T-cell subsets developed in a linear, naïve → effector → T_{EM} → T_{CM} differentiation pathway *in vivo* (62). A caveat to this study was that large numbers of adoptively transferred CD8$^+$ T-cells were needed to accurately monitor the phenotypic changes that occurred during the course of infection. It has since been suggested that this experimental system skews the development of CD8$^+$ T-cells into transitional T_{EM}, that eventually convert into T_{CM} (69). Transitional T_{EM} are thought to arise due to competition for limited antigenic stimulation available to the non-physiological number of CD8$^+$ T-cells. Under normal physiological conditions, where antigen is not limiting, it has been observed that terminally differentiated T_{EM} do not convert into T_{CM} (69). In addition, due to reduced antigenic stimulation, naïve CD8$^+$ T-cell activated late in the immune response have been found to preferentially develop into T_{CM} (70, 71). Together these findings argue that the strength of the initial activation signal modulates the progressive development of effector and memory CD8$^+$ T-cell subsets through various intermediates (16, 72). Very recent discoveries have lent additional support to this concept and suggests that cell fate can be determined early in the response, possibly even during the first cell division. Using primarily imaging studies, Chang *et al.* observed that depending on the strength of the initial stimuli, CD8$^+$ T-cells undergo asymmetrical cell division, where one daughter cell adopts a memory cell fate and the other an effector cell fate (73). An equally intriguing study, described the developmental fate of a single CD8$^+$ T-cell during the course of infection (74). Stemberger *et al.* found that the descendents of a single adoptively transferred naïve CD8$^+$ T-cell can generate the heterogeneous population of effector and memory CD8$^+$ T-cells found following infection. Taken together, these findings suggest that cell fate can be determined early in the immune response, and depending on the strength of the initial activation signal, each subsequent descendent can progress in a complex manner to generate the heterogeneity found in the CD8$^+$ T-cell population.

These considerations have been taken into account by the most recent model of CD8$^+$ T-cell generation called the "fate commitment with progressive differentiation model" ((75), Fig. 1-2*A*). Kaech and Wherry have proposed that high antigenic stimulation generates a population of short lived effector cells (SLEC), whereas low level stimulation generates memory precursor effector cells (MPEC). This differs from previous models in that MPECs acquire effector functions, but retain the capacity to become either memory cells or SLECs. The SLECs eventually die during the contraction phase, while a small number survive as terminally differentiated T_{EM}. MPECs give rise to transitional T_{EM} that progressively mature into T_{CM} that have a high proliferative potential and retain the capacity to undergo homeostatic proliferation. Therefore, this model suggests that secondary recall responses are mediated primarily by the descendents of MPECs (Fig. 1-2*B*). It remains to be seen whether this model will hold true; nevertheless, it takes into account, and may help to explain some of the disparate results in the field.

MEMORY CD8$^+$ T-CELL HOMEOSTASIS

Once established, memory CD8$^+$ T-cells are maintained at relatively constant numbers for life. Since there is no net change in the overall size of the memory pool, homeostatic proliferation of memory CD8$^+$ T-cells must be balanced by the death of excess cells (76). The longevity of memory CD8$^+$ T-cells was once thought to be mediated by periodic stimulation of the TCR with sequestered antigens or cross reactive environmental antigens (17, 77). This notion was put to rest by studies demonstrating that memory CD8$^+$ T-cells could be maintained in naïve unimmunized mice, and more importantly, in the complete absence of MHC class I (78-81). The first suggestion that soluble factors were involved in homeostasis of memory CD8$^+$ T-cells was obtained from the finding that injection of TLR ligands such as polyinosinic:polycytidylic acid (poly I:C) or lipopolysaccharide (LPS) into mice induced the antigen-independent expansion of memory phenotype (MP) CD8$^+$ T-cells (82, 83). Subsequent studies revealed that poly I:C and LPS acted indirectly through the production of type I IFNs that upregulated IL-15 production by APCs, which in turn, induced the proliferation of polyclonal MP CD8$^+$ T-cells (83, 84). The development of mice with targeted disruption of IL-15 or the IL-15 receptor α chain (IL-15Rα) provided additional evidence to support the role of IL-15 in memory CD8$^+$ T-cell homeostasis (85, 86). Mice lacking IL-15 or IL-15Rα have a normal development of CD4$^+$ T-cells but are devoid of MP CD8$^+$ T-cells, NK cells, NK T-cells, and intraepithelial lymphocytes. The absence of MP CD8$^+$ T-cells in these mice is not the result of a developmental defect, since normal MP CD8$^+$ T-cells adoptively transferred into IL-15$^{-/-}$ mice fail to survive and disappear rapidly (87). Furthermore, the direct role of IL-15 in memory CD8$^+$ T-cell homeostatic proliferation was demonstrated in IL-15 transgenic mice where overexpression of IL-15 resulted in elevated numbers of MP CD8$^+$ T-cells and the eventual development of a fatal lymphocytic leukemia (88, 89). It is now understood that under circumstances of acute infection where the pathogen and antigen are cleared, two cytokines, namely IL-15 and IL-7, are essential for maintaining the numbers of memory CD8$^+$ T-cells *in vivo* (90-92).

INTERLEUKIN-15

IL-15 was first identified by two independent groups based on its ability to mimic IL-2 induced proliferation of the IL-2 dependent T-cell line CTLL-2 (93-95). Comparisons of the primary protein and cDNA sequences of IL-15 revealed little primary homology to IL-2; however, its secondary structure places it in the four α-helix bundle cytokine family that includes other notable cytokines such as IL-2, IL-7 and IL-21 (95-97). In terms of its physical properties, IL-15 is unique in how it is expressed by APCs and recognized by responding lymphocytes. The IL-15 receptor is composed of a combination of three different subunits, α, β, and γ (Fig. 1-3*A*). The IL-15Rα chain imparts specificity for IL-15, while the IL-2/15Rβ (CD122) and common γ chain (CD132) are shared with the IL-2 receptor (98). Depending on its composition, the IL-15 receptor has varying degrees of affinity for IL-15 (99). The heterotrimeric receptor complex has high affinity for IL-15 (K_d $\sim 10^{-11}$ M), whereas the CD122/CD132 heterodimer binds IL-15 with intermediate affinity (K_d $\sim 10^{-9}$ M). Remarkably, the IL-15Rα chain alone binds to IL-15 with a high affinity equivalent to that of the heterotrimeric receptor complex. Based on its similarity to IL-2, it was at first believed that soluble IL-15 was bound by the high affinity heterotrimeric IL-15 receptor complex (100). However, it is now known that the recognition of IL-15 by CD8$^+$ T-cells can occur in the absence of IL-15Rα and requires only the expression of CD122 and CD132 (101). Interestingly, expression of IL-15Rα is absolutely required by cells other than CD8$^+$ T-cells for IL-15 to stimulate CD8$^+$ T-cells (101-104). Both IL-15 and the IL-15Rα chain must be synthesized by the same cell for functional expression of IL-15, indicating that the IL-15Rα:IL-15 complex is pre-associated prior to expression on the cell surface ((105-107), Fig. 1-3*B*). This unique mechanism of cytokine display has been termed IL-15 transpresentation (101). Such an expression modality explains why mice lacking IL-15 and IL-15Rα have virtually identical phenotypes; and why soluble IL-15 is practically undetectable in culture supernatants, and in the normal circulation (93, 108).

INTERLEUKIN-7

In addition to IL-15, IL-7 also appears to play a major role in supporting homeostasis of memory CD8$^+$ T-cells. IL-7 was originally identified as a non-redundant cytokine involved in early T-cell development; however, compelling evidence has now accumulated demonstrating the role of IL-7 in maintaining normal numbers of both naïve and memory CD8$^+$ T-cells (109-115). As previously mentioned, IL-7 is also a member of the four α-helix cytokine family and shares the CD132 receptor subunit with IL-15 and IL-2 ((98), Fig. 1-3*A*). The binding specificity for IL-7 is conferred by the IL-7Rα chain, and its expression is maintained on naïve and memory CD8$^+$ T-cells and downregulated on effector CD8$^+$ T-cells (109). Thus, MP CD8$^+$ T-cells respond well to IL-7 *in vivo*; and similar to what occurs in IL-15$^{-/-}$ mice, MP CD8$^+$ T-cells adoptively transferred into IL-7$^{-/-}$ mice fail to survive and proliferate (109). By increasing the availability of IL-7, the lack of MP CD8$^+$ T-cells in IL-15$^{-/-}$ mice, normally observed can be prevented (115). This result was demonstrated by crossing IL-15$^{-/-}$ mice to an IL-7 transgenic background. Similarly, irradiated mice deficient in only IL-15 or IL-7 can support the efficient homeostatic proliferation of adoptively transferred MP CD8$^+$ T-cells; however, the same cells fail to expand in irradiated hosts lacking both IL-15 and IL-7 (113, 114). Together, these results suggest that under lymphopenic conditions, MP CD8$^+$ T-cells can utilize either IL-15 or IL-7 to undergo homeostatic proliferation. However, under normal (i.e. non-lympohcopenic) conditions and in IL-15$^{-/-}$ mice, the level of circulating IL-7 is insufficient to maintain MP CD8$^+$ T-cell numbers. This is likely the result of competition for limited amounts of IL-7, as naive CD8$^+$ T-cells also depend on IL-7 for their survival (114). Collectively, these findings suggest that both IL-7 and IL-15 function to maintain the longevity and homeostatic proliferation of MP CD8$^+$ T-cells. The reason for the strong bias of memory CD8$^+$ T-cells towards IL-15 over IL-7 is unclear. A possible explanation could be the drastically different physical mechanisms by which the two cytokines function; IL-7 acts as a conventional soluble cytokine, whereas IL-15 is transpresented by activated APCs.

MEMORY CD8$^+$ T-CELL REACTIVATION

Upon re-exposure to a previously encountered pathogen, memory CD8$^+$ T-cells are endowed with a unique capacity to quickly respond with greater efficiency. This is the result of having enhanced TCR signaling, an ability to rapidly acquire effector function, and an abbreviated lag time prior to undergoing proliferation (116-121). Their enhanced TCR signaling capacity is a direct result of having a dense network of lipid rafts, constitutive phosphorylation of CD3ε and ζ-associated protein of 70 kDa (ZAP-70), and the ability to efficiently trigger the mitogen activated protein kinase (MAPK) signaling cascade (121, 122). Memory CD8$^+$ T-cells differ from naïve CD8$^+$ T-cells in their ability to elaborate cytolysis and effector cytokine secretion very rapidly and efficiently (116-120). Following short term (5-6hr) *in vitro* restimulation, memory CD8$^+$ T-cells can produce effector cytokines such as IFN-γ and gain the capacity to lyse target cells. Although effector CD8$^+$ T-cells can generate similar responses following *in vitro* restimulation, a defining characteristic of memory CD8$^+$ T-cells is that an overwhelming majority can produce both IFN-γ and TNF-α, and a small subset is efficient at IL-2 production (123-125). This rapid upregulation in cytokine producing capability is attributed to permanent epigenetic modifications of cytokine loci induced during priming. For example, demethylation of the IFN-γ locus in memory CD8$^+$ T-cells renders the gene accessible for immediate transcription (126). Furthermore, memory CD8$^+$ T-cells pre-synthesize and sequester some inflammatory mediators in secretory vesicles for immediate release upon TCR engagement (127, 128). Another key property of memory CD8$^+$ T-cells is their vigorous proliferative response during recall responses. In comparison to naïve CD8$^+$ T-cells, memory CD8$^+$ T-cells have a much shorter lag phase between priming and entry into cell-cycle. This is because unlike naïve CD8$^+$ T-cells that persist in G0 phase, memory CD8$^+$ T-cells exist in late G1 phase and retain preactivated factors necessary for the initiation of S phase (118). Based on these characteristics, it has been a long standing belief that memory CD8$^+$ T-cells have fewer costimulation requirements for their reactivation. However, recent studies have demonstrated that

DCs are necessary for the reactivation of memory CD8$^+$ T-cells in both peripheral tissues and in secondary lymphoid organs (129-131).. This DC requirement suggests that memory CD8$^+$ T-cells may require signals in addition to TCR stimulation for their full activation. This concept has garnered support by Borowski *et al.* who have shown that during viral infections of mice, CD28 costimulation is required for the *in vivo* reactivation of memory CD8$^+$ T-cells (132). It has been suggested that other costimulatory molecules such as 4-1BBL, a tumor necrosis family receptor (TNFR) ligand may also play a role in memory CD8$^+$ T-cell recall responses (133, 134). With the importance of memory CD8$^+$ T-cells in mediating protective immunity, further characterization of the requirements for memory CD8$^+$ T-cell restimulation is an important area of future investigation. This would include study of the restimulation requirements of secondary memory CD8$^+$ T-cells which are even more protective on a per cell basis than primary memory CD8$^+$ T-cells (135, 136). Furthering our understanding of the role of costimulation in the generation of secondary memory CD8$^+$ T-cells may have significant implications in prime-boost vaccination strategies.

MICROSPHERES AS PLATFORMS TO STUDY RECEPTOR LIGAND INTERACTIONS

Mature DC initiate CD8$^+$ T-cell responses; however, other cell types engineered to express pMHC complexes with costimulatory ligands, or equivalent surfaces constructed with purified or recombinant molecules on cell-sized microspheres can substitute for DC in these functions (137-143). Microspheres serving as artificial APC (aAPC) are useful for two purposes: (*i*) to determine the contribution of individual ligands or soluble cytokines to CD8$^+$ T cell stimulation; and (*ii*) to substitute for DCs in facilitating *in vitro* expansion of antigen specific CD8$^+$ T-cells for adoptive immunotherapy. Studies employing microspheres provided the first *in vitro* experimental evidence that activation of naïve CD8$^+$ T-cells required a signal in addition to those provided by antigen and CD28/B7 costimulation (144). Microspheres bearing immobilized pMHC and B7-1 were able to efficiently induce clonal expansion and acquisition of effector function by memory CD8$^+$ T-cells, but were ineffective in stimulating naïve CD8$^+$ T-cell responses. Upon addition of exogenous IL-12 to the cultures, naïve CD8$^+$ T-cells could be induced to expand and acquire cytolytic activity. Using the same approach it was subsequently found that type I IFNs and IL-21 could also provide the third signal necessary for naïve CD8$^+$ T-cell activation (145, 146). Thus, microspheres have proved to be very useful in determining the minimal requirements of naïve CD8$^+$ T-cell priming. Several reports have also described the successful *in vitro* expansion of antigen specific CD8$^+$ T-cells for adoptive immunotherapy using aAPCs. For example, the antigen specific human CD8$^+$ T-cells specific for hematopoietic-restricted minor histocompatibility antigens have been generated *in vitro* by microspheres bearing immobilized minor antigen pMHC complexes, B7-1 (or anti-CD28) and ICAM-1 with soluble cytokines, for immunotherapy of relapsed leukemia after allogeneic stem cell transplantation (138, 139). Microspheres are more versatile for ligand display than transfected or retrovirally transduced aAPC and may be more effective, despite not allowing ligands to move in a membrane. Although CD8$^+$ T-cells can form immune synapses involving discreet membrane localizations of receptors, these structures are not necessary for CD8$^+$ T-cell activation (147), and proliferation/differentiation occurs with ligands immobilized on cell-sized microspheres (138-146).

Many questions remain regarding relative contributions of costimulatory ligands and cytokines to CD8$^+$ T-cell priming, homeostasis, and reactivation. Our laboratory has previously used microspheres bearing pMHC complexes or MHC-like proteins to define CD8$^+$ T-cell activation requirements or to isolate and characterize NK-cell subsets, respectively (148-151). Using microspheres to dissect the activation requirements of CD8$^+$ T-cells provides several advantages. The contribution of individual ligands to various processes can be examined without interference from other ligands present on living cells. The density of each ligand can be varied precisely, much more so than cell transfectants. Ligand immobilization on microspheres as single ligands, or combinations thereof, can be performed to result in physiological ligand densities similar to those observed on APCs. For many ligands, their density can be made to even exceed physiological levels if desired, or any of the ligands can simply be titrated to specific lower densities. The ligand bearing microspheres are stable for many days at 37°C and months if stored at 4°C. Furthermore, microspheres bearing immobilized IL-15Rα/Fc can be preincubated with soluble IL-15 prior to culture so that they are able to transpresent IL-15 in a physiologically relevant manner. Thus, microspheres offer the ideal platform to examine functional receptor-ligand interactions that occur between CD8$^+$ T-cells and APCs.

RATIONALE AND OBJECTIVES

CD8$^+$ T-cells are critical for clearance of virus from an infected host. Although exquisite specificity for antigen is conferred by the TCR, several additional receptors expressed on CD8$^+$ T-cells contribute to the shaping of the CD8$^+$ T-cell response. Since the identification of IL-15, there has been intense research aimed at examining the molecular and cellular role of this cytokine. From these studies, it has become clear that IL-15 is involved in diverse aspects of CD8$^+$ T-cell activation, proliferation, and survival. A steady flow of reported new functions of IL-15 has been documented in the literature, although none more important than the discovery of the mechanism of IL-15 transpresentation by IL-15Rα. Understanding the unique mechanism of IL-15 transpresentation has provided the opportunity to reassess what was previously known about the effects of IL-15. Even to this day, much of the work on IL-15 is still being conducted using high, non-physiological concentrations of soluble IL-15, with little to no consideration of the mechanism of IL-15 transpresentation. Therefore, the first aim of this present study was to examine the singular contribution of transpresented IL-15 on CD8$^+$ T-cell responses using cell-sized microspheres bearing immobilized recombinant IL-

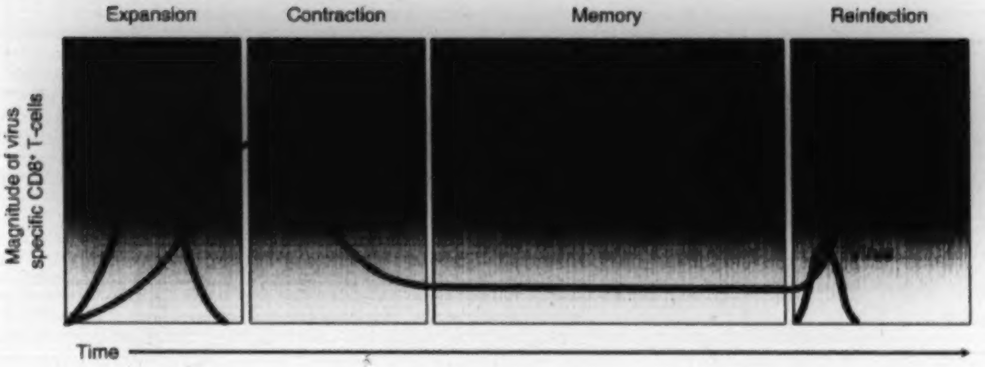
15Rα/Fc:IL-15 complexes. Using a microspheres platform, we could directly examine the contribution of transpresented IL-15, to the exclusion of other ligands.

Since soluble IL-15 has been reported to have stimulatory effects on $CD8^+$ T-cells, the second aim of this present study was to compare the stimulatory capacity of soluble versus transpresented IL-15 alone, or in combination with TCR stimulation. Interleukin-15 has been clearly demonstrated to have a role in memory $CD8^+$ T-cell homeostasis; however, its role in reactivation of memory $CD8^+$ T-cells has not been adequately addressed. Using both *in vitro* and *in vivo* approaches, the contribution of IL-15 transpresentation to memory $CD8^+$ T-cell homeostasis and restimulation was examined.

The lack of good phenotypic markers for memory $CD8^+$ T-cells continues to be one of the major obstacles in the field. Therefore, the final aim of this study was to characterize a novel monoclonal antibody (mAb) developed in our laboratory that recognizes a unique isoform of the Ly-6C molecule. The expression of Ly-6C on $CD8^+$ T-cells has been associated with a memory phenotype; however, much of the work that initially characterized Ly-6C as a memory marker was conducted prior to the development of modern methods of phenotypic assessment. Therefore, using our new anti-Ly-6C mAb iMap (immunologic memory associated protein), we reassessed the Ly-6C expression kinetics during an immune response to acute LCMV infection. Due to its unique reactivity profile, we also determined whether iMap could be used as a single marker to isolate memory $CD8^+$ T-cell populations from secondary lymphoid organs.

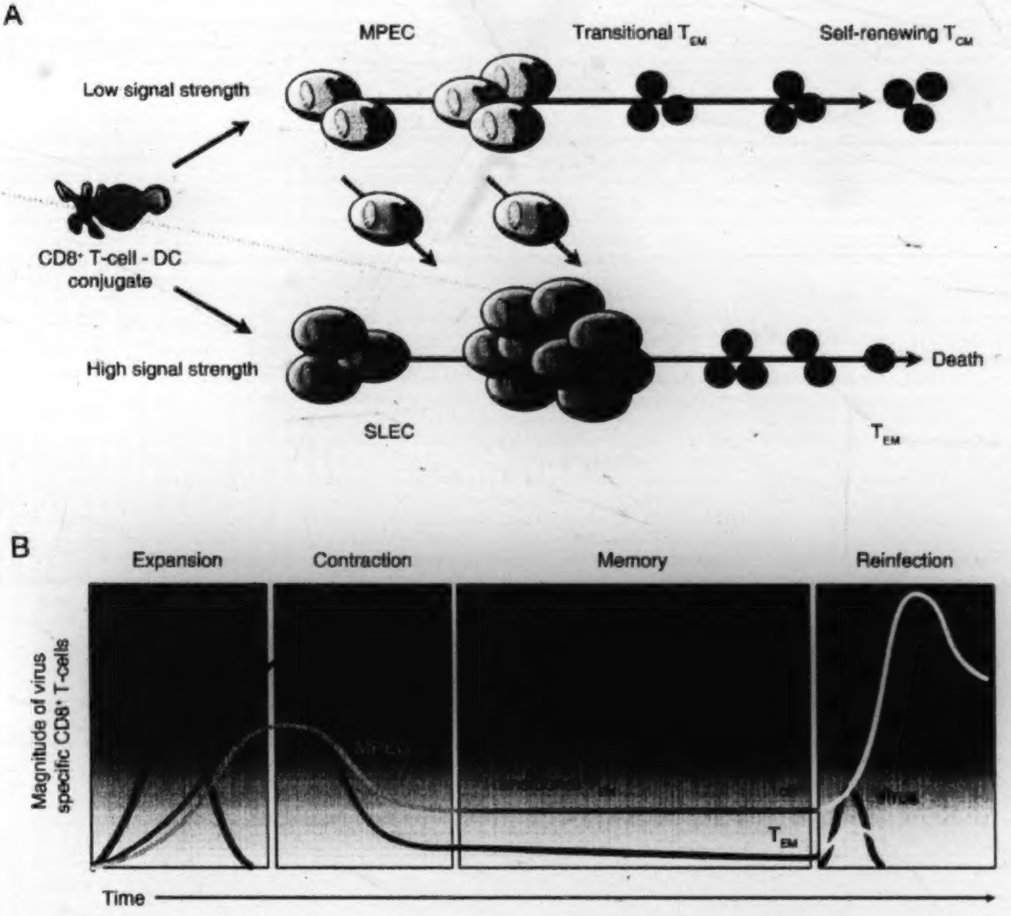
HYPOTHESES

Due to its unique mechanism of action, IL-15 transpresentation on cell-sized microspheres will provide a physiological stimulus to responding lymphocyte populations. Furthermore, since memory CD8+ T-cells are lacking in both IL-15 and IL-15Rα deficient mice, IL-15 transpresentation may preferentially affect memory CD8+ T-cell responses. Finally, owing to its restricted staining pattern, iMap will be a useful reagent for the isolation of memory CD8$^+$ T-cell populations.



adapted from Keach, S.M. and E.J. Wherry. 2007. Immunity 27:393

Figure 1-1. CD8$^+$ T-cell response to acute viral infection. During the expansion phase, activated antigen-specific CD8$^+$ T-cells undergo extensive proliferation and differentiation into effector CD8$^+$ T-cells. Upon successful clearance of the virus, the majority of the effector CD8$^+$ T-cells die by apoptosis. A small number of antigen-specific CD8$^+$ T-cells manage to survive the contraction phase and over time, progressively mature into a small population of long-lived memory CD8$^+$ T-cells. Due to their unique properties, re-exposure to the same virus is accompanied by their enhanced proliferation and the immediate clearance of the virus.


A
MPEC
Transitional T_EM
Self-renewing T_CM
Low signal strength
CD8+ T-cell - DC conjugate
High signal strength
SLEC
T_EM
Death
B
Expansion
Contraction
Memory
Reinfection
Magnitude of virus specific CD8+ T-cells
MPEC
T_EM
virus
Time

adapted from Keach, S.M. and E.J. Wherry. 2007. Immunity 27:393

Figure 1-2. Fate commitment with progressive differentiation model of memory CD8$^+$ T-cell development. A) Based on the strength of the initial activation signal, naïve CD8$^+$ T-cells differentiate into either short lived effector cells (SLEC) or memory precursor effector cells (MPEC). Strong initial activation signals generate SLECs that differentiate into end-stage T_{EM}, which have a finite lifespan. MPECs develop as a result of low antigenic stimulation, acquire effector function, and retain the capacity to become either SLECs or transitional T_{EM} that progressively develop into self-renewing T_{CM}. B) In context of an immune response, both SLECs and MPECs expand during the primary response to viral infection. Over time, MPECs gain memory CD8$^+$ T-cell potential, while SLECs do not. Protection following secondary exposure is primarily mediated by descendents of the MPECs due to either enhanced proliferative capacity.

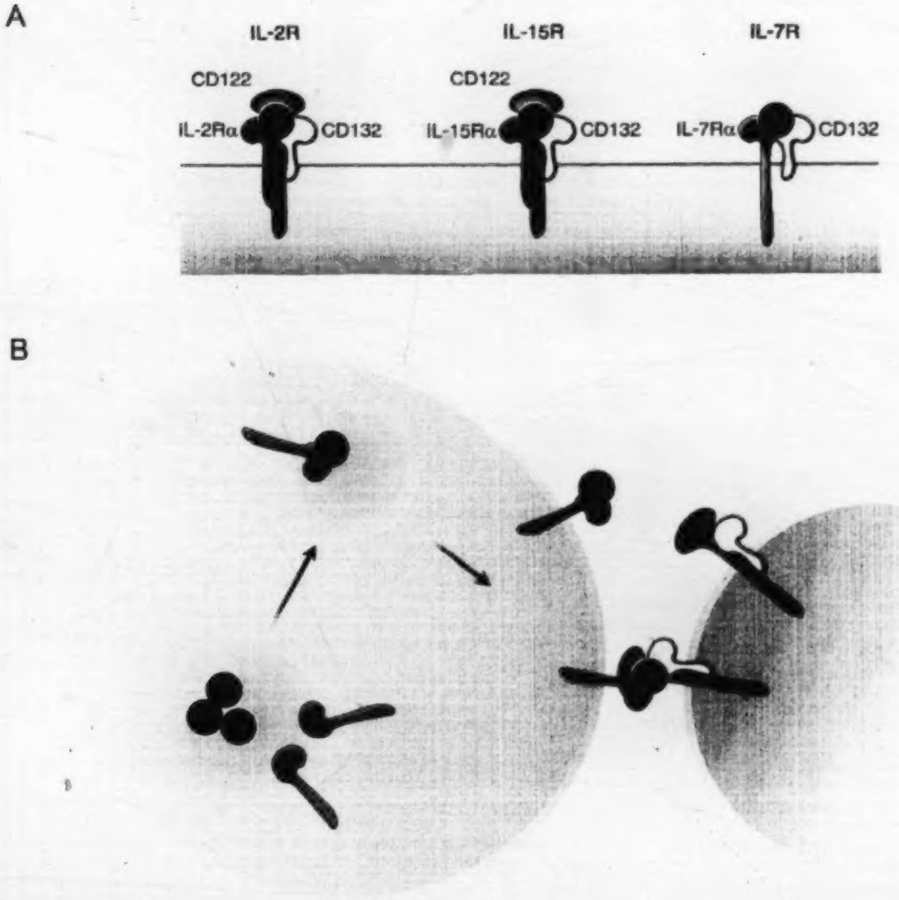


Figure 1-3. Transpresentation of IL-15 by IL-15Rα. A) Members of the four-α helix family of cytokines, IL-2, IL-15, and IL-7 all share various receptor components. Specificity for each cytokine is conferred by the individual alpha chain receptors. CD132 is shared among all three of the cytokines, whereas CD122 is shared only between IL-2 and IL-15. B) Coordinate expression of IL-15 and IL-15Rα by the same cell is required for the stabilization and cell surface expression of the IL-15Rα:IL-15 complex. Memory CD8$^+$ T-cells bearing only the CD122 and CD132 receptor components can undergo efficient homeostatic proliferation following stimulation with transpresented IL-15.

Table 1-1: Phenotypic markers associated with CD8$^+$ T-cell populations

Antigen Experienced Cells		T_N	T_{EFF}	T_{EM}	T_{CM}
CD44	Adhesion Molecule	-	+++	+++	+++
CD11a	LFA-1, α Integrin; Adhesion Molecule	+	+++	+++	+++
Ly-6C	GPI-linked; unknown function	-	+++	+++	+++
CD122	IL-2/15 receptor β chain	-	+++	+++	+++
IL-15Rα	IL-15 receptor α chain	-	++	+++	+++
Associated with Effector CD8$^+$ T-cells					
CCR5	Chemokine Receptor	-	+++	-/+	-
Perforin	Cytotoxic granule protein	-	+++	-/+	-
Granzyme B	Cytotoxic granule protein	-	+++	-/+	-
KLRG-1	killer cell lectin-like receptor G1; adhesion	-	+++	-/+	-
Dynamically Regulated during Immune Responses					
CD62L	Lymph node homing receptor	+++	-	-	+++
CCR7	Lymph node homing receptor	+++	-	-	+++
CD27	TNF receptor superfamily; costimulation	+++	-/+	-/+	+++
CD127	IL-7 receptor α chain	+++	-	-/+	+++

T_N = naïve; T_{EFF} = effector; T_{EM} = effector memory; T_{CM} = central memory CD8+ T-cell
- = not expressed; -/+ = mixed expression; + = positive expression

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CHAPTER 2

TRANSPRESENTED IL-15 DIRECTLY MEDIATES "TETHERED" ADHESION OF MEMORY CD8$^+$ T-CELLS AND MAINTAINS THEIR LONG-TERM SURVIVAL

INTRODUCTION

Interleukin-15 is a tightly regulated cytokine that plays a critical role in the development, maintenance, and activation of NK-cells and memory CD8$^+$ T-cells (1-3). It was initially thought that IL-15 mediated its activity upon binding to a heterotrimeric receptor complex composed of the IL-15Rα chain in combination with CD122 and CD132 (4-6). However, in a seminal study conducted by Dubois *et al.*, it was found that IL-15Rα has the unique ability to present IL-15 in *trans* to neighboring cells that express only CD122 and CD132 (7). This finding has since been corroborated by several studies showing that the co-ordinate expression of IL-15Rα and IL-15 by bone marrow (BM) derived cells is the primary mechanism of IL-15 action *in vivo* (8-11). Since type I IFNs and TLR ligands can upregulate both IL-15Rα and IL-15 expression in CD11c$^+$ DCs, they are likely cellular inducers of IL-15 action *in vivo* (12, 13).

Chemokines are a group of low-molecular weight proteins that rapidly transform T-cells into polarized, motile cells (14). Soluble gradients of secondary lymphoid chemokines such as CCL19 and CCL21 can induce chemoattraction and enhance transendothelial migration of lymphocytes by increasing integrin avidity (14-16). Soluble IL-15 has been shown to have similar properties, especially for NK-cells and CD8$^+$ T-cells (17-21). Due to their highly charged nature, chemokines can be captured on cell surfaces and surface-bound CCL21 has been recently demonstrated to induce "tethered" adhesion of T-cells (22). "Tethered" adhesion is the ability of surface-bound

CCL21 on APCs to mediate binding of polarized T-cells by a uropod anchor. The surface-bound chemokine induced adhesion is dependent on leukocyte function-associated antigen-1 (LFA-1) and ICAM-1/2 interactions (22). Whether IL-15 bound and transpresented by IL-15Rα can retain its chemokine-like properties and enhance adhesion of lymphocytes in a similar manner is currently unknown.

In the present study, we demonstrate that IL-15 bound and presented on microspheres by immobilized IL-15Rα/Fc has a rapid and profound effect on the selective binding of lymphocyte populations. Within two to four hrs, spleen cells bind to microspheres bearing the IL-15Rα/Fc:IL-15 complex, forming aggregates. Upon examination of microsphere bound spleen cells, we found that there was a preferential binding of $CD8^+$ T-cells, which corresponded to higher expression levels of CD122. Furthermore, within the $CD8^+$ T-cell population, there was preferential binding of T_{CM} $CD8^+$ T-cells as compared to their naïve and T_{EM} counterparts. Live-cell imaging demonstrated that binding of $CD8^+$ T-cells to microspheres bearing transpresented IL-15 resembled "tethered" adhesion mediated by surface-bound chemokines. Since IL-15 is necessary for the maintenance of memory $CD8^+$ T-cells, we hypothesized that transpresented IL-15 adhesion may have functional consequences for the responding $CD8^+$ T-cells. We found that *ex vivo* LCMV-specific memory $CD8^+$ T-cells could be maintained by IL-15Rα/Fc:IL-15 microspheres *in vitro* for over two months while preserving antigen specificity. Taken together, our findings suggest that transpresented IL-15 in the absence of any additional molecules, functions as a novel adhesion molecule, in addition to its more well-known role as a homeostatic cytokine.

MATERIALS AND METHODS

REAGENTS

Recombinant mouse IL-15Rα/Fc chimera and polyclonal goat antiserum against mouse IL-15Rα and mouse IL-15 were purchased from R&D Systems (Minneapolis, MN). Recombinant mouse IL-15, IFN-γ and granulocyte macrophage-colony stimulating factor (GM-CSF) was obtained from Peprotech Inc., (Rocky Hill, NJ). LPS from *Escherichia coli* 0111:B4 was acquired from Sigma-Aldrich (Oakville, ON, Canada). The H-2D^b restricted LCMV glycoprotein (gp) 33-41 (KVATFATM) and influenza A/PR8 nucleoprotein (NP) 366-374 (ASNENMETM) peptides were purchased from BIOpeptide Co., (San Diego, CA). R-PE labeled H-2D^b-gp33 and H-2D^b-NP366 tetramers were prepared by the Canadian Network for Vaccines and Immunotherapeutics (CANVAC) core facilities (Montréal, QC, Canada). The unconjugated, biotinylated or fluorochrome-conjugated forms of the following mAbs were purchased from eBioscience (San Diego, CA), BD Biosciences (Mississauga, ON, Canada) or Invitrogen (Burlington, ON, Canada): 53-6.7 and 5H10, anti-CD8α; GK1.5, anti-CD4; MB19-1, anti-CD19; N418, anti-CD11c; TM-β1, anti-CD122; 4G3, anti-CD132; PK136, anti-NK1.1; 145-2C11, anti-CD3ε; IM7, anti-CD44; XMG1.2 and R4-6A2, anti-IFN-γ; and MEL-14, anti-CD62L. Anti-CD132, 4G3, and polyclonal goat antiserum against mouse IL-15 and IL-15Rα were conjugated to Alexa 488 or Alexa 647 using mAb conjugation kits from Invitrogen.

MICE AND LCMV INFECTIONS

Eight to twelve week old C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were infected by *i.p.* injection of 2×10^5 plaque forming units (PFU) of LCMV-Armstrong harvested from infected BHK-21 monolayers (LCMV-Armstrong was a gift from Dr. Pamela Ohashi, University of Toronto, Toronto, ON, Canada). LCMV infected mice were housed in biocontainment facilities. All animal studies followed the guidelines of the Canadian Council on Animal Care and the University of Alberta Health Animal Policy and Welfare Committee.

MICROSPHERE PREPARATION

Microspheres were prepared using either 4.5μm tosylactivated M-450 Dynalbeads or 5μm sulfate-modified polystyrene microspheres from Invitrogen. IL-15Rα/Fc immobilization on 4.5μm tosylactivated Dynalbeads was performed as follows: 1×10^7 microspheres were incubated with 1μg of IL-15Rα/Fc in a final volume of 100μL in phosphate buffered saline (PBS) at 37°C with rotation for 30 minutes (min). Following immobilization, 1% bovine serum albumin (BSA)/PBS was added to final concentration of 0.5% (w/v) and incubated for an additional 16-24 hours (hr) at 37°C with rotation. Immobilization of IL-15Rα/Fc onto 5μm sulfate-modified polystyrene microspheres was performed with slight modifications due to increased immobilization efficiency compared to the tosylactivated M-450 Dynalbeads. To maintain similar IL-15Rα/Fc density, 0.25μg of IL-15Rα/Fc was immobilized onto 1×10^7 microspheres in a total volume of 1mL PBS at 4°C with rotation for 15 mins. Following immobilization, 1% BSA/PBS was added to final concentration of 0.5% weight to volume ratio (w/v) and incubated for an additional 30 mins at 4°C with rotation. Microspheres were washed with 0.1% BSA/PBS and in some instances, incubated with 100ng of IL-15 overnight at 4°C with rotation. Following IL-15 loading, microspheres were washed extensively with 0.1% BSA/PBS and resuspended in culture medium. 1μg of 145-2C11 (anti-CD3ε) was immobilized onto 1×10^7 5μm sulfate-modified polystyrene microspheres as outlined above. Density of immobilized IL-15Rα/Fc or transpresented IL-15 on microspheres was analyzed by flow cytometry using ligand specific antibodies.

BONE MARROW DERIVED DENDRITIC CELL PROPAGATION

Bone marrow was harvested from the femurs and tibias of C57BL/6 mice and resuspended in culture media composed of RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 0.055mM β-2-mercaptoethanol (2-ME) and 20ng/mL mouse GM-CSF. Ten million BM cells in 50mL of 20ng/mL GM-CSF supplemented culture medium was plated in suspension culture plates. On day 3 of culture, 50mL of fresh 20ng/mL GM-CSF supplemented culture media was added to each plate. Non- and semi-adherent cells were harvested and resuspended at $1x10^6$ cells/mL in culture media supplemented with 10ng/mL GM-CSF. Fifty million day 6 cells were plated in tissue culture treated culture plates overnight. The following day, non-adherent BM derived DCs (BMDC) were harvested. For activation, non-adherent day 7 BMDCs were cultured in tissue culture treated plates in the presence of 60ng/mL LPS and 20ng/mL IFN-γ, and activated BMDCs were then harvested the following day.

AGGREGATION ASSAY

Spleens were isolated from C57BL/6 mice and gently disrupted with a tissue homogenizer. Spleen cell suspensions were depleted of erythrocytes with 0.15M NH_4Cl, washed and resuspended in culture media. One half million microspheres were cultured with $0.25x10^6$ spleen cells in a final volume of 250μL in 48-well tissue culture plates. In some instances, neutralizing antibodies were added to a final concentration of 0.25μg/mL at the start of the culture. Images were captured at various time points using an inverted microscope with an attached Retiga Q-image charge-coupled device camera using Openlab software (Improvision, Waltham, MA).

TRANSPRESENTED IL-15 MICROSPHERE BASED CELL SEPARATION

Two million erythrocyte depleted C57BL/6 spleen cells were cultured with $4x10^6$ paramagnetic IL-15Rα/Fc:IL-15 microspheres in a final volume of 1mL in 2mL microfuge tubes. At the indicated time points, tubes were placed into a magnetic rack to separate IL-15Rα/Fc:IL-15 microspheres and bound cells, from unbound spleen cells. Bound and unbound spleen cells were stained for various cell surface markers, fixed with 4% paraformaldehyde and analyzed by flow cytometry.

LIVE-CELL TIME-LAPSE MICROSCOPY

$CD8^+$ T-cells were cultured with blue fluorescent microspheres with immobilized IL-15Rα/Fc:IL-15 in glass chamber slides (Sigma-Aldrich). Slides were cultured for 4 hrs at 37°C to initiate cell aggregate formation. Live cell time-lapse microscopy was performed with a Zeiss LSM510 confocal microscope with a 37°C heated stage. A plan-Apochromat 40x/1.3 oil differential interference contrast objective lens was used. Images were acquired in 15 second intervals and combined with Imaris software (Bitplane, Saint Paul, MN) to generate a time lapse video.

ISOLATION AND FLOW CYTOMETRIC SORTING OF EX VIVO $CD8^+$ T-CELLS

Spleens and LNs (axillary, brachial, inguinal, and superficial cervical) were isolated from C57BL/6 mice and gently disrupted with a tissue homogenizer. $CD8^+$ T-cell isolations were performed using an EasySep $CD8^+$ T-cell enrichment kit (StemCell Technologies Inc., Vancouver, BC, Canada). In some instances, negatively enriched $CD8^+$ T-cells were further stained with FITC conjugated anti-CD8α and R-PE labeled H-$2D^b$/gp33 tetramers and sorted into tetramer positive and negative $CD8^+$ T-cell populations using a BD FACSAria (BD Biosciences). Analysis of flow cytometric data was conducted using BD FACSDiva or FCS Express (De Novo Software, Los Angeles, CA).

$CD8^+$ T-CELL STIMULATION WITH IL-15Rα/FC MICROSPHERES

H-$2D^b$/gp33 tetramer positive $CD8^+$ T-cells were cultured with paramagnetic IL-15Rα/Fc:IL-15 microspheres at a 2:1 microsphere to cell ratio in 24-well flat-bottomed tissue culture plates (Corning, Inc., Corning, NY). Cultures were incubated at 37°C with 5% CO_2 with weekly replacement of culture media and microspheres. At the indicated time points, cells were harvested and analyzed by flow cytometry.

IFN-γ ELISPOT ASSAY

Ten million EL4 target cells were pulsed with 8μg of either LCMV gp33 or influenza NP366 peptide for 1h at 37°C in FCS and washed extensively prior to use. On days 35 and 77 of culture, IL-15 maintained $CD8^+$ T-cells were incubated with peptide pulsed EL4 target cells at a 1:2 or 1:5 effector to target cell ratio (E:T) in a ninety-six-well MultiScreen-HA plate (Millipore, Bedford, MA) coated with anti-mouse IFN-γ (mAb: AN-18) for 5h at 37°C. At the end of the incubation period the ELISPOT plate was washed and then biotin-conjugated anti-mouse IFN-γ (mAb: R4-6A2) was added followed by horseradish peroxidase (HRP) conjugated-streptavidin (Jackson ImmunoResearch Laboratory, West Grove, PA). The plates were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Sigma-Aldrich), and the IFN-γ spots were enumerated with a Bioreader-4000 (BioSys, Karben, Germany).

RESULTS

RAPID AGGREGATE FORMATION OF SPLEEN CELLS WITH IL-15 TRANSPRESENTING MICROSPHERES

Initially we were interested in utilizing IL-15 transpresentation as a means to present cytokines on cell-sized microspheres for use in expansion of $CD8^+$ T-cells for immunotherapeutic approaches. We found that if IL-15Rα/Fc was co-immobilized with other T-cell ligands in the presence of IL-15, it had profound effects on the binding and aggregation of lymphocytes. We therefore set out to examine this process further, and prepared microspheres with immobilized IL-15Rα/Fc, in the absence of any additional immobilized ligands (Fig. 2-1). IL-15 transpresentation by immobilized IL-15Rα/Fc was accomplished by the pre-incubation of IL-15Rα/Fc microspheres with IL-15 followed by extensive washing to remove unbound soluble IL-15. To provide a physiological stimulus, the density of immobilized IL-15Rα/Fc and IL-15 was adjusted to a level similar to that found on LPS and IFN-γ activated BMDCs. Following their preparation, IL-15Rα/Fc microspheres with or without preloaded IL-15 were cultured with spleen cell preparations. Microspheres bearing immobilized IL-15Rα/Fc in the absence of IL-15 were virtually ignored when cultured with spleen cells (Fig. 2-2). In contrast, when IL-15 was pre-incubated with the IL-15Rα/Fc microspheres, there was a rapid and profound formation of microsphere-spleen cell aggregates, which remained stable for at least 24 hrs. Our results therefore demonstrate that physiological densities of transpresented IL-15 on microspheres can mediate the binding and aggregation of spleen cells. This binding occurs in the complete absence of any additional immobilized ligands, and requires the presence of both immobilized IL-15Rα/Fc and preloaded IL-15.

Since the high-affinity IL-15 receptor is a heterotrimeric complex consisting of the shared CD122 and CD133 subunits and the private IL-15Rα chain, we sought to determine whether we could inhibit aggregation with neutralizing antibodies against the various receptor components. Aggregation could be clearly inhibited upon addition of a polyclonal neutralizing antibody against IL-15 (Fig. 2-3). The addition of a neutralizing polyclonal antibody against IL-15Rα also inhibited aggregation, although this appeared to be the result of microsphere crosslinking. This observation does not exclude the possibility the polyclonal anti-IL-15Rα could also inhibit aggregation by blocking IL-15Rα expressed by the responding spleen cells. Monoclonal antibodies directed against the shared IL-15 receptor components CD122 and CD132 also completely inhibited aggregate formation. Aggregation was unaffected by the addition of isotype control antibodies (Fig. 2-3, bottom panels). Taken together, our results demonstrate that the IL-15 transpresentation by microspheres results in rapid binding and aggregation with spleen cell preparations that requires the functional expression of CD122, CD132 and possibly IL-15Rα by the responding lymphocytes.

CD8⁺ T-CELLS PREFERENTIALLY BIND TO TRANSPRESENTED IL-15 DUE TO ELEVATED EXPRESSION LEVELS OF IL-15 RECEPTOR COMPONENTS

Since we found that spleen cells could bind and aggregate with IL-15 transpresenting microspheres, we next assessed if there were specific lymphocyte populations that preferentially bound to the microspheres. We therefore developed a paramagnetic microsphere based cell separation assay to distinguish between the microsphere bound versus unbound populations. Paramagnetic microspheres were prepared with immobilized IL-15Rα/Fc and preloaded with IL-15. Spleen cells were cultured with IL-15Rα/Fc:IL-15 microspheres in 2mL microfuge tubes and at various time points the tubes were placed into a magnetic tube rack. Microspheres and bound cells were magnetically held against the tube walls, while unbound spleen cells remained free in the culture media or at the bottom of the tube. Culture media containing the unbound cells was removed to a separate tube and the bottoms of the tubes rinsed with fresh culture media, making sure to avoid the microspheres and bound cells held against the tube walls. The unbound and microsphere bound cells were counted and subsequently stained for cell surface markers to distinguish the various lymphocyte populations.

The paramagnetic based cell separation demonstrated that within 1 hr, a small percentage of spleen cells bound to IL-15 transpresenting microspheres (Fig. 2-4*A*). Maximal binding of approximately 30% of spleen cells was detected by 4 hrs of culture. Flow cytometric analysis of the initial spleen cell population demonstrated that they were composed of approximately 60% B-cells, 20% CD4⁺ T-cells, 15% CD8⁺ T-cells and 3% NK-cells (Fig. 2-4*B*). Following 4 hrs of culture, analysis of bound and unbound populations revealed that CD4⁺ T-cells comprised similar proportions of the bound and unbound population at 17% and 19%, respectively. In contrast, B-cells comprised a reduced proportion of the bound population at 44% compared to 69% in the unbound population. CD8⁺ T-cells were enriched in the bound population where they made up 30% of the bound population, whereas they only comprised 6% of the unbound population. Although not as pronounced, there is a slightly increased proportion of NK cells in the bound versus unbound population. When examined as a fold change from the original splenic population, CD8$^+$ T-cells were the only population that had a dramatic two-fold increase in number of cells bound to IL-15 transpresenting microspheres (Fig. 2-4*C*). Therefore, our findings suggest that CD8$^+$ T-cells preferentially bound transpresented IL-15. It should be noted that in the normal splenic architecture, lymphocytes are partitioned into discrete microdomains, so our *in vitro* assay using splenic preparations may underestimate the ability of CD8$^+$ T-cells to bind to transpresented IL-15 on the surface of APCs (23). For example, within LNs B-cell populate the cortex and medulla, whereas the paracortical region is the T-cell rich area (24). In the spleen, T-cells populate the periarteriolar lymphoid sheaths and B-cells reside in the lymph follicles and marginal zone. Therefore, *in vivo*, T-cells and B-cells likely do not compete for the same cellular source of transpresented IL-15 within secondary lymphoid organs.

Since spleen cell binding to IL-15 transpresenting microspheres could be inhibited by the addition of neutralizing antibodies against the various IL-15 receptor components (Fig. 2-3), we hypothesized that differential expression of the various IL-15 receptor components could be mediating the preferential binding of CD8$^+$ T-cells. Therefore, we performed multi-parameter flow cytometric analysis to examine the cell surface expression patterns of IL-15Rα, CD122 and CD132 on the various lymphocyte populations (Fig. 2-4*D*). Low expression of IL-15Rα was detected on all lymphocyte populations examined and the lowest mean fluorescence intensity (MFI) of IL-15Rα expression was found on NK-cells. This is in contrast to what has been previously demonstrated in the literature; however, in our hands we have not been able to demonstrate any significant expression of IL-15Rα on cells other than activated CD11c$^+$ BMDCs (25). This may be due to differences in the method of detection since we were using a polyclonal antibody against IL-15Rα, whereas reports of IL-15Rα expression in various lymphocytes was conducted using an IL-15/Fc fusion protein. Regardless, in contrast to IL-15Rα expression, there were relative differences in CD122 expression by the various lymphocyte populations. B-cells and CD4$^+$ T-cells expressed low levels of CD122, whereas a proportion of CD8$^+$ T-cells expressed CD122 and half of the NK-cells expressed CD122. When CD132 expression levels were examined, we found a slightly elevated expression on CD8$^+$ T-cells and relatively low expression on the other lymphocytes. Taken together with our previous findings that antibodies against CD122 and CD132 can inhibit aggregation (Fig. 2-3), our results suggest that the increased level of CD122 and CD132 found on CD8$^+$ T-cells may account for their preferential binding to IL-15 transpresenting microspheres. Unfortunately, the expression levels of CD122 on the bound and unbound populations could not be assessed due to its rapid internalization upon cytokine binding (26, 27).

CENTRAL MEMORY PHENOTYPE CD8$^+$ T-CELLS PREFERENTIALLY BIND MICROSPHERES TRANSPRESENTING IL-15

Since only a small proportion of CD8$^+$ T-cells expressed elevated levels of CD122, we next wanted to determine if subpopulations within the total CD8$^+$ T-cell pool preferentially bound to transpresented IL-15. CD8$^+$ T-cells from unimmunized mice contain both naïve and MP CD8$^+$ T-cells that are specific for environmental antigens. CD44 and CD62L expression can be used to delineate these CD8$^+$ T-cells into naïve, T_{CM} and T_{EM} populations. Naïve CD8$^+$ T-cells are typically CD44^{low}CD62L^{high}, whereas antigen experienced cells express high levels of CD44 and can be defined as T_{CM} and T_{EM} populations based on CD62L expression. T_{CM} CD8$^+$ T-cells express high levels of CD62L, whereas T_{EM} CD8$^+$ T-cells express low levels of CD62L. Initial examination of the original spleen cells demonstrated that CD8$^+$ T-cells comprised ~15% of the total cells and of those CD8$^+$ T-cells, 70% of the cells were of a naïve phenotype, 17% were T_{CM} and 9% were T_{EM} (Fig. 2-5*A*). Following 4 hrs of culture with the IL-15 transpresenting microspheres, the bound and unbound CD8$^+$ T-cells were analyzed for CD44 and CD62L expression. The most significant difference between the two populations occurred in the naïve and T_{CM} $CD8^+$ T-cell populations. There was a decreased percentage of naïve phenotype $CD8^+$ T-cells and a corresponding increase in the proportion of T_{CM} $CD8^+$ T-cells in the bound $CD8^+$ T-cell population. This finding suggested that there is a measurable preference for the binding of T_{CM} $CD8^+$ T-cells compared to naïve or T_{EM} $CD8^+$ T-cells to transpresented IL-15.

We next analyzed the expression of IL-15 receptor components on the $CD8^+$ T-cell subpopulations by flow cytometry. Expression of IL-15Rα and CD132 was negligible on the total $CD8^+$ T-cell population and a small proportion of $CD8^+$ T-cells expressed CD122 (Fig. 2-5*B*). Following gating of the $CD8^+$ T-cell subpopulations using CD44 and CD62L expression, we found that naïve $CD8^+$ T-cells expressed low levels of IL-15Rα and CD122; however, they did express slightly higher levels of CD132. In contrast, T_{EM} $CD8^+$ T-cells expressed increased levels of IL-15Rα and CD122 with slightly lower CD132 expression compared to naïve $CD8^+$ T-cells. Analysis of T_{CM} $CD8^+$ T-cells demonstrated that they expressed similar levels of IL-15Rα and CD132 as T_{EM} $CD8^+$ T-cells, but they had significantly increased expression of CD122. Taken together, our results demonstrate that $CD8^+$ T-cells preferentially bind to IL-15 transpresenting microspheres and within the bound $CD8^+$ T-cell pool, T_{CM} $CD8^+$ T-cells are more prevalent than T_{EM} and naïve $CD8^+$ T-cells. Our findings suggest that differential expression of IL-15 receptor components may help explain why memory $CD8^+$ T-cells are more dependent on IL-15 for their survival (28-31).

IL-15 TRANSPRESENTATION MEDIATES POLARIZATION AND "TETHERED" ADHESION OF $CD8^+$ T-CELLS

Since we have demonstrated that IL-15 transpresentation by microspheres mediates aggregation and preferential binding of $CD8^+$ T-cells, we wanted to examine the interactions at the more closely using live-cell imaging using fluorescent microspheres bearing IL-15Rα/Fc:IL-15 complexes. When purified $CD8^+$ T-cells were cultured with blue fluorescently labeled microspheres transpresenting IL-15, we found that the aggregates were very dynamic (Movie 2-1). The aggregates were composed of rapidly moving CD8$^+$ T-cells that appeared to be tumbling around a core of microspheres. The majority of interactions appeared to occur between CD8$^+$ T-cells and the microspheres, and not between the CD8$^+$ T-cells themselves. Interestingly, unbound CD8$^+$ T-cells maintained a round morphology; whereas, the majority of microsphere bound CD8$^+$ T-cells had a polarized, amoeboid appearance with a distinct uropod and leading edge. When individual conjugates were examined, CD8$^+$ T-cells were attached to the blue fluorescent microspheres transpresenting IL-15 by their uropod, while their leading edge remained highly active (thick arrow panel 1, Fig. 2-6). In contrast, CD8$^+$ T-cells bound to anti-CD3ε coated non-fluorescent microspheres formed tight conjugates with very little movement (thin arrow panel 1, Fig. 2-6). The polarized morphology of the CD8$^+$ T-cells induced by transpresented IL-15 on microspheres resembled "tethered" adhesion mediated by surface-bound chemokines (22). "Tethered" adhesion was defined as the ability of lymph node APCs to present surface bound chemokines to T-cells resulting in a polarized amoeboid T-cell adhered by its uropod. This has been suggested to capture and prime T-cells for synapse formation. Friedman *et al.* demonstrated that this chemokine mediated adhesion was dependent on LFA-1 and ICAM-1/2 interactions. However, the microspheres used in our study do not have any additional immobilized adhesion ligands; therefore, IL-15 transpresentation alone can mediate this "tethered" adhesion. Taken together, our observations suggest that transpresented IL-15 can act as an adhesion molecule, while inducing rapid movement and polarization of CD8$^+$ T-cells.

IL-15 TRANSPRESENTATION SUSTAINS LONG-TERM SURVIVAL AND ANTIGEN SPECIFICITY OF CD8$^+$ T-CELLS IN VITRO

IL-15 is required for the homeostatic proliferation of memory CD8$^+$ T-cell survival *in vivo.* Since memory CD8$^+$ T-cells preferentially bound to IL-15 transpresenting microspheres, we wanted to determine if the IL-15 transpresenting microspheres could maintain memory CD8$^+$ T-cells *in vitro*, in the absence of additional stimuli. To address this, we immunized C57BL/6 mice with LCMV-Armstrong and allowed sufficient time for a LCMV specific memory CD8$^+$ T-cell population to develop *in vivo*. LCMV gp33 specific CD8$^+$ T-cells were then sorted from the spleen and LNs using H-2D^b/gp33 tetramers. The gp33-specific CD8$^+$ T-cells were cultured with paramagnetic IL-15Rα/Fc:IL-15 microspheres at a 2:1 microsphere to cell ratio, with weekly replacement of the microspheres and culture medium. At the indicated time points, small aliquots of the cells were removed and analyzed by flow cytometry for tetramer binding and phenotypic markers. Following sorting, approximately 0.25x10^6 CD8$^+$ T-cells were obtained with 90% of them being H-2D^b/gp33 tetramer positive (Fig. 2-7*A*). By day 7 of culture the initial population had expanded to approximately 1x10^6 CD8$^+$ T-cells with 61% of them staining with the H-2D^b/gp33 tetramer. From day 7 to day 77 of culture, the tetramer positive population remained constant at ~60%. The initial reduction in tetramer positive cells was either due to an out-growth of tetramer negative cells, or due to some level of non-specific tetramer binding during the original sorting procedure. In spite of the initial reduction, from day 7 to day 77 of culture, the tetramer positive population in the IL-15 maintained CD8$^+$ T-cells remained constant. Upon analysis of the phenotype of the tetramer positive cells, the initial CD8$^+$ T-cells were 65% T_{EM} and 35% T_{CM} (Fig. 2-7*B*). At day 35 of culture, these cells had a similar make up of T_{EM} to T_{CM} CD8$^+$ T-cells. However, by day 77 of culture, the phenotypes of the tetramer positive cells had reversed such that 64% of the cells were T_{CM} while 34% were T_{EM}. Whether the change in phenotype was due to a conversion of T_{EM} to T_{CM} or whether there was an outgrowth of T_{CM} CD8$^+$ T-cells during the culture period is unknown.

Since the CD8$^+$ T-cells maintained their tetramer binding ability, we next sought to examine the functionality of the antigen-specific CD8$^+$ T-cells upon recognition of peptide pulsed target cells in a IFN-γ ELISPOT assay (Fig. 2-7*C*). The ELISPOT assay requires very few cells and was therefore well suited to measuring the functionality of the transpresented IL-15 maintained CD8$^+$ T-cells. Small numbers of transpresented IL-

15 maintained CD8$^+$ T-cells were removed on day 35 and 77 and cultured with EL4 target cells pulsed with either LCMV gp33 or control Influenza NP366 peptides in ninety-six-well plates coated with anti-IFN-γ mAbs. Following 5 hrs of culture, the plates were washed and analyzed for bound IFN-γ. We found that culture of the CD8$^+$ T-cells with Influenza NP366 pulsed EL4 targets resulted in no IFN-γ spots whereas, culture of LCMV gp33 EL4 targets resulted in a significant amount of IFN-γ secretion. There was a slightly increased level of IFN-γ production by day 77 cultured CD8$^+$ T-cells versus the day 35 culture. Whether this was due to the differences in memory cell composition of the two populations is unknown. Taken together, these findings suggest that microsphere transpresented IL-15 alone is sufficient to maintain the long-term survival and antigen-specificity of CD8$^+$ T-cells *in vitro*. In addition, although the IL-15 transpresenting microspheres did not induce significant amounts of proliferation, they could maintain the relative numbers of tetramer positive T-cells for the duration of culture. Therefore, IL-15 transpresenting microspheres may provide utility for the maintenance of *in vitro* expanded antigen specific CD8$^+$ T-cells for use in adoptive immunotherapy.

DISCUSSION

This study describes a previously unknown role of transpresented IL-15 as a potent adhesion ligand for CD8$^+$ T-cells. We have shown that IL-15 presented by immobilized IL-15Rα/Fc on cell-sized microspheres, in the absence of any additional ligands, can mediate the preferential binding of CD8$^+$ T-cells. Differential expression levels of CD122 correlated with the preferential binding of CD8$^+$ T-cells and the T_{CM} CD8$^+$ T-cell subset. Importantly, transpresented IL-15 adhesion is similar to adhesion mediated by surface-bound chemokines (22). Friedman *et al.* demonstrated that when the secondary lymphoid chemokine CCL21 is bound and presented on the cell surface of APCs, it mediates the binding of T-cells in a polarized, highly motile manner, dependent on LFA-1 and ICAM-1/2 interactions. Woolf *et al.* subsequently provided contradictory evidence demonstrating that surface-bound chemokines induced robust T-cell motility, and only upon application of shear-stress, would T-cells undergo integrin mediated adhesion (32). We have demonstrated in the present study that transpresented IL-15 in shear-stress free conditions, directly mediates CD8$^+$ T-cells adhesion and robust motility, in the complete absence of any additional adhesion ligands. It would therefore be interesting if transpresented IL-15 also has differential effects on T-cell motility and adhesion under flow, or in extravascular shear-stress free conditions. Furthermore, as IL-15 has been reported to induce LFA-1 activation on NK-cells and CD8$^+$ T-cells, it would be interesting to examine what effect co-immobilized ICAM-1 would have on transpresented IL-15 mediated adhesion (19, 21).

A second major discovery by Friedman *et al*. was that the adhesion mediated by surface-bound chemokines preceded TCR signaling, and that chemokine-tethered T-cells were hyper-responsive to subsequent contacts with APCs. Whether this is true for transpresented IL-15 is not known, however, we have demonstrated that transpresented IL-15 is sufficient for the long-term survival and maintenance of memory CD8$^+$ T-cells. It is not inconceivable that transpresented IL-15 could initiate T-cell activation since soluble IL-15 and TCR crosslinking induce highly similar gene expression patterns in memory CD8$^+$ T-cells (33). Serial contacts with IL-15 transpresenting APCs may therefore prime CD8$^+$ T-cells for subsequent TCR induced activation. In addition to CD8$^+$ T-cells, NK-cells are also dependent on IL-15 derived signals for their development and survival (28, 29). We have shown here that NK-cells also express elevated levels of CD122 and it is likely that NK-cells are able to bind transpresented IL-15 when present in sufficient quantities. In support of this concept, transpresentation of IL-15 by CD11c$^+$ DCs was recently demonstrated to be required for NK-cell priming *in vivo* (34). This interaction required TLR stimulation of NK-cells and the upregulation of IL-15Rα and IL-15 on DCs by type I IFNs.

By mediating the preferential binding of memory CD8$^+$ T-cells, IL-15 transpresentation by DCs may play an important role in regulation of immune activation, particularly during recall responses to previously encountered antigens. This may occur through several different, but interconnected mechanisms. One possibility is that by providing a hierarchy of interaction, IL-15 transpresentation may mediate early interaction of activated DCs with memory CD8$^+$ T-cells and possibly NK-cells. Early interactions with NK-cells would facilitate their rapid priming and subsequent migration to sites of antigenic challenge. In addition, by interacting with memory CD8$^+$ T-cells, it would potentially enable rapid recall responses by giving antigen experienced CD8$^+$ T-cells priority in sampling antigen presented by the DC. If cognate pMHC is not found by the responding CD8$^+$ T-cell, IL-15 transpresentation would provide the minimal stimuli necessary for maintaining the survival of the CD8$^+$ T-cell. Additionally, the enhanced motility induced by transpresented IL-15 stimulation could increase the probability of memory CD8$^+$ T-cells to successfully interact with a nearby DC bearing cognate pMHC. In a situation where the TCR engagement occurs, IL-15 transpresentation may augment the response since soluble IL-15 has been reported to enhance CD8$^+$ T-cell activation (35-38). Whether IL-15 transpresentation can augment CD8$^+$ T-cell activation similar to soluble IL-15 in currently unknown, but will be a focus of future investigation (Chapter 3). This may occur through costimulating TCR signaling or through IL-15 mediated upregulation of integrin adhesion (19, 21). By limiting IL-15 transpresentation to times of inflammation, transpresented IL-15 may play a critical role in regulating complex lymphocyte interactions with DCs.

Professional APCs such as DCs initiate CD8$^+$ T cell responses; however, other cell types engineered to express peptide class I MHC complexes and co-stimulator ligands, or equivalent surfaces constructed with recombinant molecules on cell-sized microspheres, can substitute for DCs in these functions (39-45). Since microspheres are incapable of secreting cytokines, pro-inflammatory cytokines that are required for optimal CD8$^+$ T-cell activation must be exogenously added to *in vitro* cultures.

Transpresentation of IL-15 may therefore enhance the utility of microspheres as artificial antigen presenting surfaces by fostering binding and interaction of CD8$^+$ T-cells with microspheres, and possibly providing activation signals. Furthermore, IL-15 transpresentation by microspheres may be useful for maintaining large numbers of expanded antigen specific CD8$^+$ T-cells *in vitro* prior to adoptive immunotherapeutic approaches.

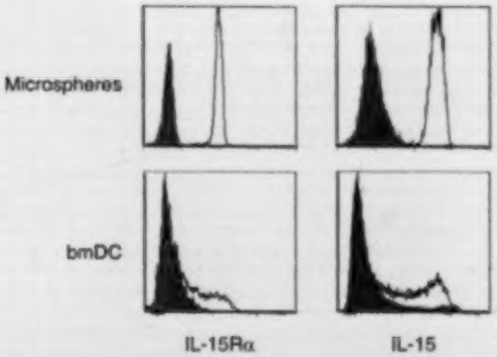


Figure 2-1. IL-15 transpresentation by microspheres and bone marrow derived dendritic cells. 1μg of IL-15Rα/Fc was immobilized onto 1x10^7 paramagnetic microspheres and preloaded with 100ng of IL-15. Day 6 BMDCs were activated overnight with LPS and IFN-γ. Microspheres and BMDC were stained with fluorochrome conjugated antibodies against IL-15Rα and IL-15 and analyzed by flow cytometry. IL-15Rα and IL-15 expression by BMDC was gated on CD11c$^+$ cells. Shaded histograms represent staining with isotype controls.

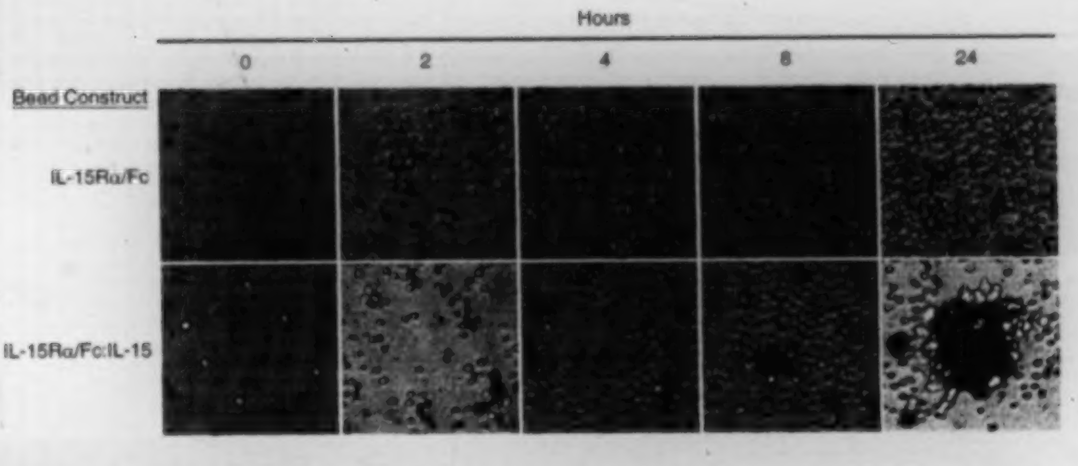
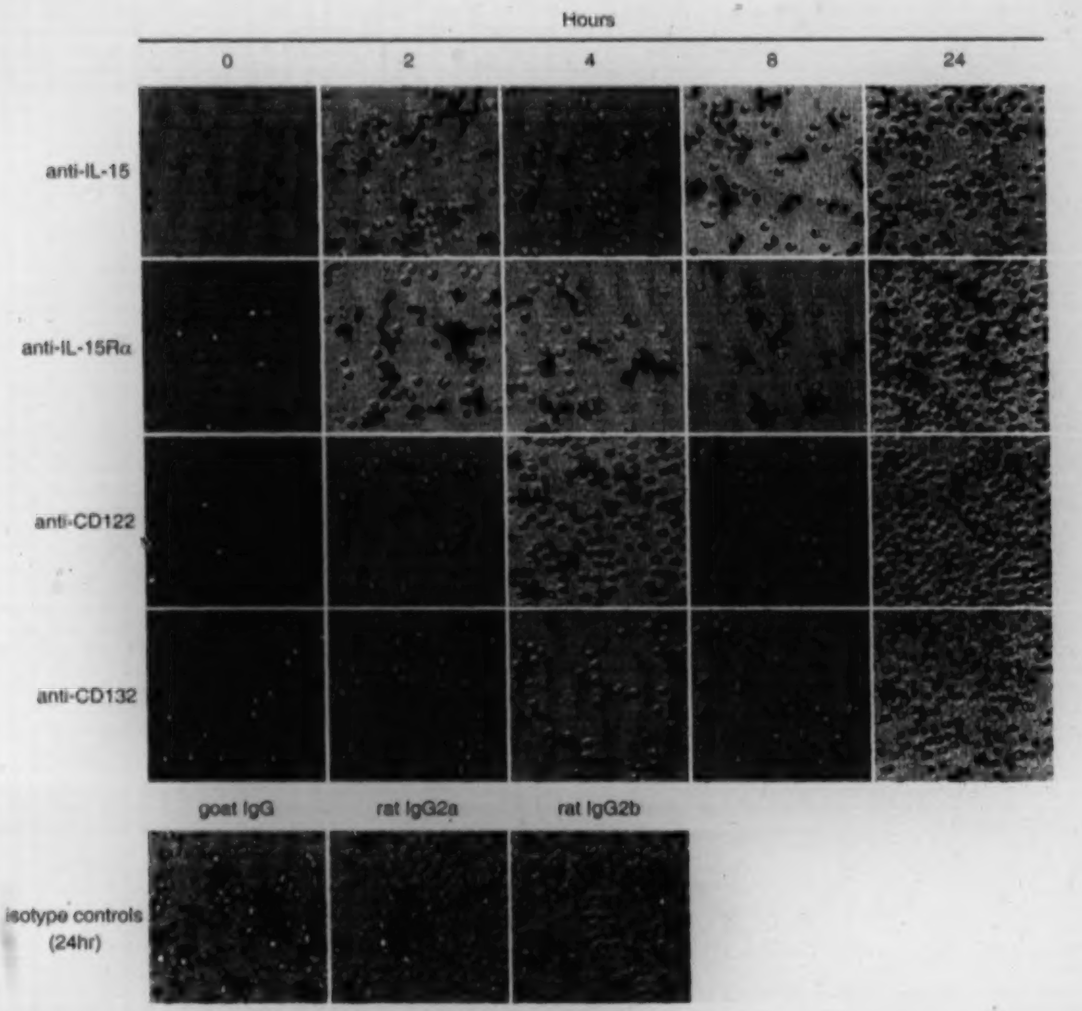

Hours
0
2
4
8
24
Bead Construct
IL-15Rα/Fc
IL-15Rα/Fc:IL-15

Figure 2-2. Aggregation of spleen cells with IL-15α microspheres requires the presence of IL-15. A) Kinetics of spleen cell aggregation with paramagnetic microspheres bearing immobilized IL-15Rα/Fc with our without preloaded IL-15.


Hours
0
2
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8
24
anti-IL-15
anti-IL-15Rα
anti-CD122
anti-CD132
goat IgG
rat IgG2a
rat IgG2b
isotype controls
(24hr)

Figure 2-3. Expression of IL-15 receptor components is required for adhesion to IL-15 transpresenting microspheres. Neutralizing antibodies against IL-15, IL-15Rα, CD122 and CD132 inhibit aggregation of spleen cells with IL-15 transpresenting microspheres. Isotype controls (rIgG2a, rIgG2b, and polyclonal goat IgG) do not inhibit aggregation.

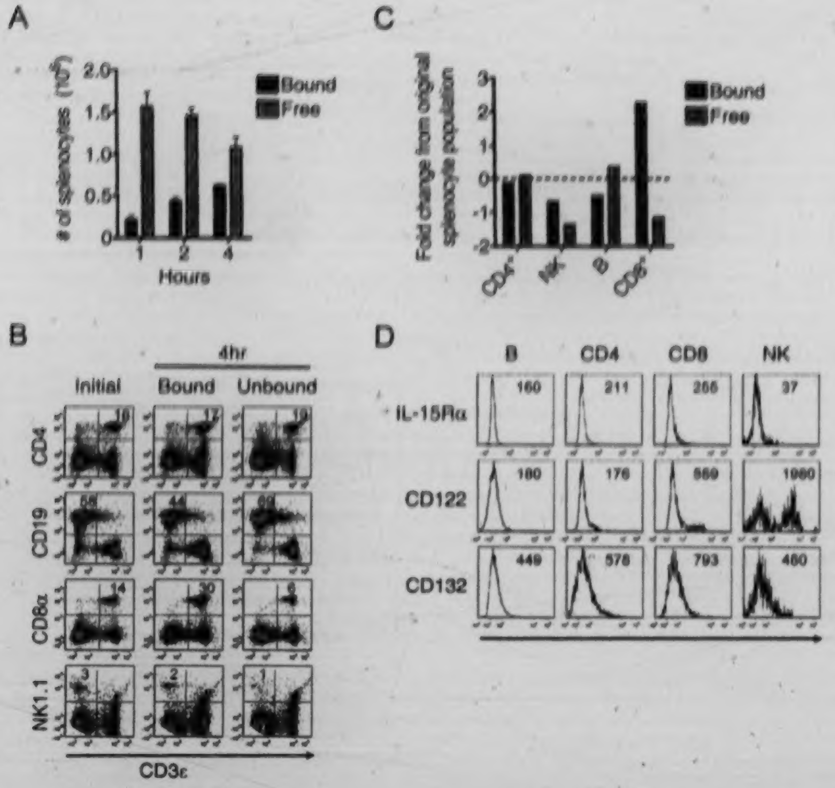

Figure 2-4. CD8$^+$ T-cells and NK-cells express elevated levels of CD122 and preferentially bind transpresented IL-15 on microspheres. Erythrocyte depleted spleen cells were cultured with paramagnetic microspheres bearing immobilized IL-15Rα/Fc and preloaded IL-15. At the indicated time points microsphere bound and unbound cells were separated by placing the cultures into a magnet. A) Microsphere bound and unbound cells were counted using a haemocytometer at the indicated time points. B) Flow cytometric analysis of initial, bound and unbound spleen cells following 4 hrs of culture. C) Fold change of bound and unbound populations compared to original spleen cell population. D) Expression of IL-15 receptor components by the gated spleen cell populations. Numbers indicate MFI of staining with the indicated antibodies. One representative experiment of three is shown.

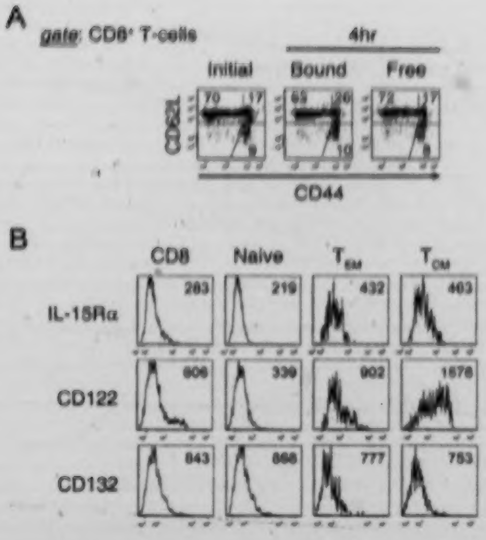

Figure 2-5. Central memory CD8$^+$ T-cells express elevated levels of CD122 and preferentially bind transpresented IL-15 on microspheres. CD8$^+$ T-cells were cultured with paramagnetic microspheres bearing immobilized IL-15Rα/Fc and preloaded IL-15. A) At the indicated time points microsphere bound and unbound CD8$^+$ T-cells were analyzed for the expression of CD62L and CD44. B) Expression of IL-15 receptor components by the CD8$^+$ T-cell sub-populations. Numbers indicate MFI of staining with indicated antibodies. One representative experiment of three is shown.

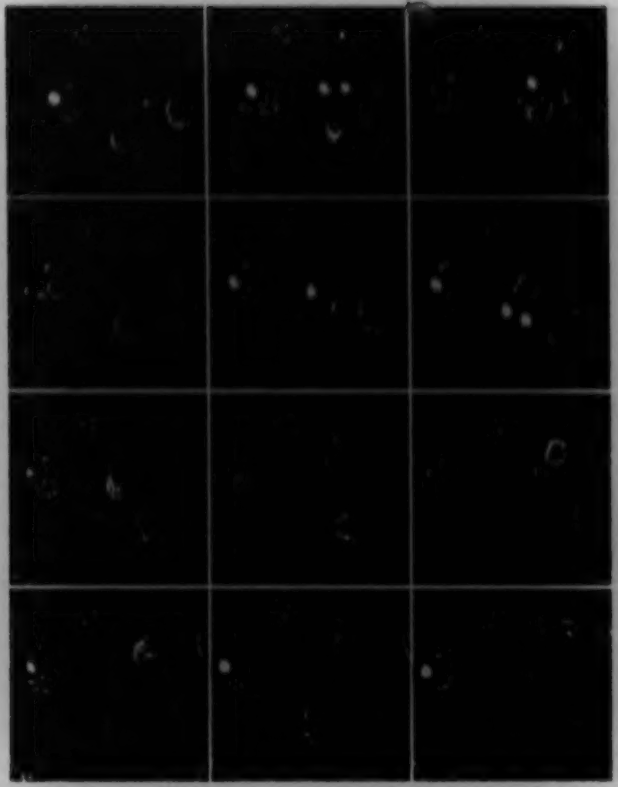

Figure 2-6. IL-15 transpresentation mediates "tethered" adhesion of CD8$^+$ T-cells. CD8$^+$ T-cells were cultured together with blue fluorescent microspheres bearing immobilized IL-15Rα/Fc preloaded with IL-15 (thick arrow) and non-fluorescent microspheres coated with anti-CD3ε (thin arrow). CD8$^+$ T-cells and microspheres were cultured in chamber slides and analyzed using a Zeiss LSM510 confocal microscope with a 37°C heated stage. Each numbered panel represents an image acquired in 15 second intervals.

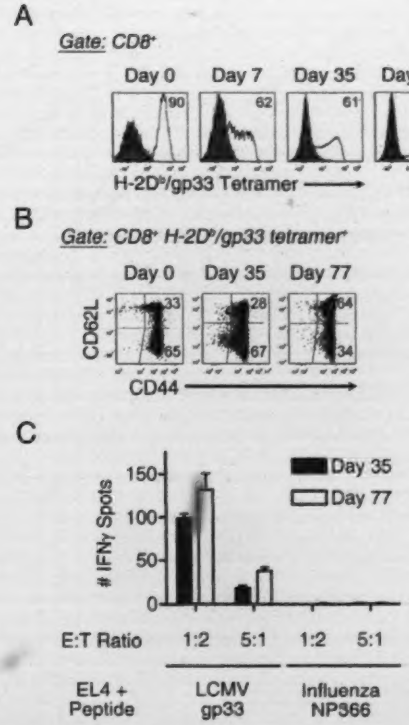

Figure 2-7. IL-15 transpresentation can sustain long-term antigen specificity and survival of memory CD8$^+$ T-cells *in vitro*. H-2D^b/gp33 tetramer positive memory CD8$^+$ T-cells were cultured with IL-15 transpresenting microspheres with weekly replacement of paramagnetic IL-15Rα/Fc:IL-15 microspheres for a period of 77 days. A) Tetramer staining of transpresented IL-15 maintained LCMV gp33 specific memory CD8$^+$ T-cells. Open histograms represent staining with H-2D^b/gp33 tetramers. Shaded histograms represent staining with H-2D^b/NP366 tetramers. B) CD62L and CD44 expression by H-2D^b/gp33 tetramer positive CD8$^+$ T-cells at the indicated time points. C) IFN-γ ELISPOT analysis of transpresented IL-15 maintained CD8$^+$ T-cells. EL4 target cells were pulsed with either LCMV gp33 or Influenza NP366 peptide. Day 35 and 77 CD8$^+$ T-cells were cultured with peptide pulsed EL4 target cells at the indicated E:T ratios.

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CHAPTER 3

INTERLEUKIN-15 TRANSPRESENTATION AUGMENTS CD8$^+$ T-CELL ACTIVATION AND IS REQUIRED FOR OPTIMAL RECALL RESPONSES BY CENTRAL MEMORY CD8$^+$ T-CELLS

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INTRODUCTION

Interleukin-15 is a member of the four α-helix family of cytokines and has diverse roles in lymphocyte development, homeostasis, and activation (1-3). Both IL-15 and IL-15Rα deficient mice have similar phenotypes consisting of severe defects in the development of NK, NK T-cells, intraepithelial lymphocytes, and a lack of peripheral MP CD8$^+$ T-cells (4, 5). In spite of these defects, the initiation of primary CD8$^+$ T-cell responses against LCMV occurs in mice lacking IL-15 or IL-15Rα expression; however, the long term maintenance and homeostatic proliferation of memory CD8$^+$ T-cells is critically dependent on IL-15 derived signals (4-8). Consistent with these findings, *in vivo* administration or transgenic overexpression of IL-15 results in enhanced proliferation and increased numbers of MP CD8$^+$ T-cells (9-11).

Since the identification of IL-15, a significant amount of data supporting its role as a T-cell activator has accumulated due to structural and functional similarities to IL-2 (12, 13). *In vitro* studies have shown that high concentrations of IL-15 can induce cellular proliferation and transcription of effector molecules such as IFN-γ and granzyme (grB) (14, 15). In addition, soluble IL-15 can augment anti-CD3ε induced activation and cytokine production of both mouse and human CD8$^+$ T-cells (16-19). Whether IL-15 synergizes with, or acts independently of TCR stimulation is presently unclear, however stimulation of MP CD8$^+$ T-cells by IL-15 or anti-CD3ε induces remarkably similar gene expression patterns, as identified by cDNA microarray analysis (20).

It was initially thought that IL-15 mediated its effects upon binding to a heterotrimeric receptor complex composed of IL-15Rα, CD122 and CD132 (21, 22). It was later revealed that CD8$^+$ T-cells did not require IL-15Rα expression and could respond to IL-15 when expressing only CD122 and CD132 (23). Upon further analysis, co-ordinate expression of IL-15Rα and IL-15 by BM derived cells was found to be crucial for IL-15 mediated effects on CD8$^+$ T-cells *in vivo* (23-26). Unlike other soluble cytokines that induce signals upon binding to their respective receptors, IL-15 bound to its specific high affinity IL-15Rα chain can be retained on the cell surface and presented in *trans* to neighboring cells expressing only CD122 and CD132 (27). Due to the extremely high affinity of the IL-15 and·IL-15Rα interaction (K_d=38pM), it has been suggested that the receptor-cytokine complex may act as a membrane bound stimulatory molecule in a contact dependent manner (28). Taken together, these findings suggest that a BM derived cell capable of expressing both IL-15Rα and IL-15 could regulate memory CD8$^+$ T-cell responses. Since DCs can express both IL-15Rα and IL-15 following activation, they are likely candidates involved in IL-15 transpresentation (29). In support of this concept, DC derived IL-15 is essential for the induction of delayed-type hypersensitivity responses in mice (30).

Requirements for the initiation of primary CD8$^+$ T-cell responses have been well documented; however, the events required for the initiation of recall responses by memory CD8$^+$ T-cells are less well defined. Similar to naive CD8$^+$ T-cells, recall responses by memory CD8$^+$ T-cells to previously encountered antigens requires the presence of DCs (31). Cell-sized microspheres have been successfully used to investigate the role of immobilized protein ligands on lymphocyte activation and/or adhesion (32-37). Substitution of microspheres for DCs allows for the precise control of the constellation and density of ligands displayed to a responding CD8$^+$ T-cell. Using microspheres as a platform to generate antigen presenting surfaces, we sought to determine the specific role of IL-15 transpresentation in the reactivation of MP and *ex vivo* antigen specific memory CD8$^+$ T-cells. Since DCs themselves respond to IL-15 through enhanced survival, upregulation of co-stimulatory molecules, and production of effector cytokines, microspheres allow a focus on the direct effects of IL-15 transpresentation on the responding CD8$^+$ T-cells, in the absence of other stimulatory signals (29, 38-40).

In the present study, we demonstrate that transpresented IL-15 in combination with a TCR stimulus provided by either anti-CD3ε or pMHC was significantly more effective at inducing proliferation and upregulating IFN-γ and grB expression by CD8$^+$ T-cells than soluble IL-15. In addition, co-immobilization of anti-CD3ε and transpresented IL-15 was more effective than either anti-CD3ε or transpresented IL-15 alone, or anti-CD3ε and transpresented IL-15 provided on two separate surfaces. In agreement with previous studies, we found that transpresented IL-15 preferentially stimulated MP CD8$^+$ T-cells; however, in pursuing this further we find that T_{CM} CD8$^+$ T-cells were more responsive to IL-15 transpresentation than T_{EM} CD8$^+$ T-cells *in vitro*. Following antigen specific pMHC stimulation, T_{CM} CD8$^+$ T-cells were also more dependent on transpresented IL-15 than T_{EM} CD8$^+$ T-cells for the induction of grB and proliferation *in vitro*. Upon examination *in vivo*, LCMV specific T_{CM} CD8$^+$ T-cells had reduced proliferative ability following LCMV infection in IL-15Rα deficient hosts, whereas T_{EM} CD8$^+$ T-cell proliferation was less affected by the absence of IL-15Rα. Thus, T_{CM} CD8$^+$ T-cells are more responsive to transpresented IL-15 alone; and in the context of TCR stimulation, require IL-15 transpresentation for the induction of optimal recall responses. Our findings distinguish the role of IL-15 transpresentation in stimulation of CD8$^+$ T-cell subsets and have implications for the *ex vivo* reactivation and expansion of antigen experienced CD8$^+$ T-cells for adoptive immunotherapy.

MATERIALS AND METHODS

REAGENTS

Recombinant mouse IL-15Rα/Fc chimera, polyclonal goat anti-mouse IL-15Rα and polyclonal goat anti-mouse IL-15 were purchased from R&D Systems (Minneapolis, MN). Recombinant mouse IL-15 was obtained from Peprotech Inc., (Rocky Hill, NJ). BD DimerX H-2D^b/Ig recombinant mouse fusion protein was acquired from BD Biosciences (Mississauga, ON, Canada). H-2D^b restricted LCMV gp33-41 (KVATFATM) and influenza A/PR8 NP366-374 (ASNENMETM) peptides were purchased from BIOpeptide Co., (San Diego, CA). R-PE labeled H-2D^b-gp33 and H-2D^b-NP366 tetramers were prepared by the CANVAC core facilities (Montréal, QC, Canada). 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was purchased from Invitrogen Corp., (Burlington, ON, Canada). The unconjugated or fluorochrome-conjugated forms of the following mAbs; 145-2C11, anti-CD3ε; 53-6.7 and 5H10, anti-CD8α; IM7, anti-CD44; MEL-14, anti-CD62L; GB12, anti-grB; and XMG1.2, anti-IFN-γ were purchased from eBioscience (San Diego, CA) or Invitrogen. FITC-conjugated goat anti-hamster IgG and R-PE-conjugated donkey anti-goat IgG F(ab$'$)$_2$ fragments were obtained from Jackson ImmunoResearch Laboratory (West Grove, PA). Anti-H-2D^b; B22.249, was purified from hybridoma supernatants using protein G affinity chromatography and conjugated to fluorescein isothiocyanate (FITC) using a FITC protein labeling kit from Invitrogen.

MICE AND LCMV INFECTIONS

Eight to twelve week old C57BL/6J, B6129SF2/J and B6129X1Il15ra^{tm1Ama}/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were infected by *i.p.* injection of 2x10^5 PFU of LCMV-Armstrong harvested from infected BHK-21 monolayers (LCMV-Armstrong was a gift from Dr. Pamela Ohashi, University of Toronto, Canada). B6129X1Il15ra^{tm1Ama}/J and B6129SF2/J mice were maintained in specific pathogen free conditions prior to use, and all LCMV infected mice were housed in biocontainment facilities. All animal studies followed the guidelines of the Canadian Council on Animal Care and the University of Alberta Health Animal Policy and Welfare Committee.

ISOLATION, CFSE LABELING AND FLOW CYTOMETRIC SORTING OF EX VIVO CD8⁺ T-CELLS

Mice were anesthetized with halothane (Sigma-Aldrich, Oakville, ON, Canada) and euthanized by cervical dislocation. Spleens and LNs (axillary, brachial, inguinal, and superficial cervical) were isolated, pooled and gently disrupted with a tissue homogenizer. $CD8^+$ T-cell isolations were performed using an EasySep $CD8^+$ T-cell enrichment kit (StemCell Technologies Inc., Vancouver, BC, Canada). For CFSE labeling, $CD8^+$ T-cells were washed with 0.1% BSA (Sigma-Aldrich) in PBS (Invitrogen) and resuspended at a density of $5x10^6$ cells/mL in 0.1% BSA/PBS containing a final concentration of 2 or 5μM CFDA-SE for 5 mins at 37°C, followed by washing with 2% FBS (Hyclone, Logan, UT) in PBS. In some instances, negatively enriched, CFSE labeled $CD8^+$ T-cells were further sorted into naïve, T_{CM} and T_{EM} populations based on CD8α, CD44 and CD62L expression. Purity of each population was typically >95% following sorting with a BD FACSAria (BD Biosciences). Due to limited numbers of cells available and technical limitations, all experiments were conducted using $CD8^+$ T-cells enriched from pooled spleens. In most cases, enriched $CD8^+$ T-cells were divided among the experimental groups in triplicate. Although experimental samples were performed in triplicate, the true sample size is 1.

MICROSPHERE PREPARATION

Microsphere constructs were prepared by incubating $1x10^7$ 5μm sulfate modified polystyrene microspheres (Invitrogen) with 0.1 - 1μg of various proteins at 4°C with rotation for 15 mins in PBS. Unbound sites on the microspheres were blocked with the addition of 1% BSA/PBS followed by an additional 30 min incubation at 4°C with rotation. Microspheres were washed with 0.1% BSA/PBS and resuspended in culture medium. For peptide loading of immobilized H-2D^b/Ig, 1x10^7 microspheres were incubated with 20μg of peptide in 100μL FBS for 1 hr at 37°C, washed extensively with 0.1% BSA/PBS and resuspended in culture medium. In some instances, 1x10^7 microspheres were incubated with 100ng of recombinant mouse IL-15 overnight at 4°C with rotation and washed extensively prior to use with 0.1% BSA/PBS. Unless otherwise stated, 1μg of IL-15Rα/Fc was immobilized alone or in combination with 0.1μg of anti-CD3ε or 1μg of H-2D^b/Ig onto 1x10^7 microspheres. Density of immobilized proteins or transpresented IL-15 on microspheres was analyzed by flow cytometry using ligand specific antibodies.

CD8$^+$ T-CELL STIMULATION WITH MICROSPHERES

Two hundred and fifty thousand CD8$^+$ T-cells or sorted CD8$^+$ T-cell populations were cultured with 0.5x10^6 microspheres in ninety-six-well flat-bottomed culture plates (Corning Inc., Corning, NY), in a final volume of 0.25mL. Culture medium consisted of RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 0.055mM 2-ME (Invitrogen). Cultures were incubated at 37°C with 5% CO_2 and harvested at the indicated time points for flow cytometric analysis. Where indicated, IL-15 was added at a final concentration of 0 – 100ng/mL at the start of culture.

CFSE DILUTION AND INTRACELLULAR CYTOKINE ANALYSIS

Brefeldin A (Invitrogen) was added to cultures at a final concentration of 1μg/mL for 4 hrs prior to harvest. CFSE labeled CD8$^+$ T-cells were harvested and stained with fluorochrome-conjugated mAbs against cell surface markers, fixed and permeabilized with BD Cytofix and Cytoperm buffers (BD Biosciences), and counter stained with fluorochrome-conjugated anti-IFN-γ and anti-grB mAbs. All staining and fixation steps were performed at 4°C for 15 mins. Flow cytometric acquisition was performed using a BD FACSCalibur, FACSCanto or FACSAria. >30,000 gated events were acquired for each sample. Flow cytometric analysis was conducted using FCS Express software (DeNovo Software, Thornhill, ON, Canada). Percent positive calculations were performed using Overton Subtraction in FCS Express (41).

ADOPTIVE TRANSFERS

Negatively enriched CD8$^+$ T-cells from B6129SF2/J mice infected *i.p.* with LCMV-Armstrong forty days prior were CFSE labeled and sorted into naïve, T_{EM}, and T_{CM} populations, as previously described. A half million of each T-cell population was transferred by *i.v.* injection into naïve B6129SF2/J or B6129X1Il15ra^{tm1Ama}/J mice. The following day, recipient mice were infected *i.p.* with LCMV-Armstrong as previously described. Four days post infection, recipient mice were euthanized and spleens and LNs were harvested. Single cell suspensions of spleen and lymph node samples were prepared and stained with anti-CD8α and analyzed for CFSE dilution.

RESULTS

IL-15 AUGMENTATION OF ANTI-CD3ε INDUCED CD8$^+$ T-CELL ACTIVATION IS MORE EFFECTIVE IN THE PRESENCE OF IL-15Rα/FC

Although exogenous IL-15 has been shown to augment T-cell responses following TCR stimulation (16-19), to our knowledge no direct comparison of soluble IL-15 versus transpresented IL-15 has been examined in the presence of a TCR stimulus. In addition, previous reports have used total mouse splenocyte preparations or human peripheral blood mononuclear cells (PBMC) for the measurement of CD8$^+$ T-cell responses, which may include cells expressing IL-15Rα and hence capable of transpresenting IL-15. Therefore, we sought to directly compare soluble IL-15 versus transpresented IL-15 in augmenting anti-CD3ε induced activation of purified C57BL/6 CD8$^+$ T-cells. To address this issue we utilized a 5μm microsphere platform to control and manipulate the stimulatory signals that *ex vivo* CD8$^+$ T-cells would receive. Microspheres were prepared with either anti-CD3ε or IL-15Rα/Fc immobilized alone, or co-immobilized together and analyzed by flow cytometry (Fig. 3-1*A*). The density of anti-CD3ε could be preserved when IL-15Rα/Fc was co-immobilized, thereby maintaining microspheres offering equivalent TCR stimulatory signals with or without IL-15Rα/Fc. To potentially boost sensitivity for the detection of IL-15 mediated stimulation, anti-CD3ε was immobilized at a density that induced sub-optimal T-cell proliferation (Fig. 3-1*B*). Once prepared, the various microsphere constructs were incubated with CD8$^+$ T-cells in the presence of increasing concentrations of soluble IL-15. In the absence of immobilized IL-15Rα/Fc, soluble IL-15 would act directly on the responding CD8$^+$ T-cells. In contrast, IL-15 in the presence of IL-15Rα/Fc could be transpresented by the immobilized receptor due to the high affinity of the IL-15Rα/Fc for IL-15 (28). CD8$^+$ T-cells were cultured with the various microspheres for 24 or 48 hrs and analyzed for expression of intracellular IFN-γ and grB, or for cell division detected by CFSE dilution.

Following culture of CD8$^+$ T-cells with BSA or IL-15Rα/Fc microspheres in the absence of soluble IL-15, no induction of IFN-γ, grB or proliferation was noted (Fig. 3-1*B*). Minimal induction of CD8$^+$ T-cell responses was also detected in the absence of soluble IL-15 following stimulation with either anti-CD3ε, or anti-CD3ε and IL-15Rα/Fc microspheres, thus confirming the sub-optimal density of immobilized anti-CD3ε. Upon addition of increasing concentrations of soluble IL-15 to BSA or IL-15Rα/Fc microsphere cultures, minimal increases in intracellular IFN-γ and grB expression and proliferation were detected in the responding CD8$^+$ T-cells (Fig. 3-1*B*). In comparison, soluble IL-15 could augment anti-CD3ε induced IFN-γ and grB expression, and cellular proliferation (Fig. 3-1*B*). However, in the presence of co-immobilized anti-CD3ε and IL-15Rα/Fc, IFN-γ and grB expression and proliferation of responding CD8$^+$ T-cells was induced to substantially higher levels at all IL-15 concentrations examined (Fig. 3-1*B*). These findings demonstrate that approximately five-fold higher concentrations of soluble IL-15 are required to induce similar levels of proliferation, IFN-γ and grB production with immobilized anti-CD3ε, compared to when IL-15Rα/Fc is co-immobilized with anti-CD3ε.

SYNERGISM OF IL-15 TRANSPRESENTATION AND ANTI-CD3ε ON CD8^{+} T-CELL STIMULATION

To investigate the direct ability of transpresented IL-15 to augment anti-CD3ε induced proliferation and cytokine production in light of our preceding findings, microspheres were prepared with co-immobilized anti-CD3ε and IL-15Rα/Fc in the presence or absence of preloaded IL-15. To ensure that IL-15 available in the culture was transpresented in the context of IL-15Rα/Fc, microspheres were incubated overnight with IL-15 and subsequently washed extensively to remove any soluble unbound IL-15. To verify IL-15 was indeed transpresented by our microsphere constructs, they were stained and analyzed by flow cytometry (Fig. 3-2*A* and *B*). The microspheres allowed for the precise titration of transpresented IL-15, as demonstrated by the tight correlation between the density of immobilized IL-15Rα/Fc and bound IL-15 shown as individual histograms (Fig. 3-2*A* middle and right panels), or as plots of the MFI of anti-CD3ε, IL-15Rα/Fc, and IL-15 versus the concentration of immobilized IL-15Rα/Fc on the microspheres (Fig. 3-2*B*). A critical factor was the ability to maintain a constant density of immobilized anti-CD3ε, while accurately titrating the density of immobilized IL-15Rα/Fc and transpresented IL-15 (Fig. 3-2*A* and *B*).

Following incubation for 24 or 48 hrs, CD8^{+} T-cells cultured with IL-15Rα/Fc microspheres in the absence of IL-15 did not express IFN-γ or grB, nor did they undergo any detectable cell division (Fig. 3-2*C*). Anti-CD3ε co-immobilized with any concentration of IL-15Rα/Fc in the absence of IL-15 induced a low but consistent level of cell division and cytokine production. Microspheres transpresenting higher densities of IL-15 were able to induce grB expression and proliferation in a small number of CD8^{+} T-cells that was almost equivalent to anti-CD3ε stimulation. However, when IL-15 bound to immobilized IL-15Rα/Fc was co-presented with anti-CD3ε, there was a dramatic dose dependent increase in cell division and IFN-γ and grB expression (Fig. 3-2C). Based upon these findings, transpresented IL-15 greatly enhances anti-CD3ε induced CD8$^+$ T-cell activation in a dose dependent manner. In addition, the augmentation mediated by transpresented IL-15 appeared to be synergistic since the responses to combined TCR and transpresented IL-15 stimuli were not merely a sum of the individual responses.

CD8$^+$ T-CELL RESPONSES OCCUR WHEN IL-15 AND ANTI-CD3ε ARE PRESENTED ON TWO SEPARATE SURFACES WHILE CO-PRESENTATION OF IL-15 AND ANTI-CD3ε PROVIDES OPTIMAL STIMULATION

We next determined whether IL-15 transpresentation could augment an anti-CD3ε stimulus provided on a separate surface, or whether the two signals must be presented on the same surface. To this end, microspheres were prepared with immobilized IL-15Rα/Fc alone (Rα), or in combination with anti-CD3ε in the presence or absence of preloaded IL-15 (2C11Rα and 2C11Rα15, respectively). BSA blocked microspheres served as a negative control. Anti-CD3ε was immobilized at a sub-optimal density as previously demonstrated (Fig. 3-1 and 3-2) and IL-15Rα/Fc was immobilized at a high density to provide optimal IL-15 stimulation. The various microspheres were cultured individually or in combination with CFSE labeled C57BL/6 CD8$^+$ T-cells for 24 and 48 hrs and subsequently analyzed for proliferation and intracellular IFN-γ and grB production by flow cytometry. When CD8$^+$ T-cells were cultured with each microsphere construct individually, BSA and Rα microspheres had no effect on any of the responses examined (Fig. 3-3). Rα15 microsphere stimulation of CD8$^+$ T-cells resulted in no IFN-γ production and a low level of grB expression and proliferation. 2C11Rα microspheres induced a low level of proliferation, IFN-γ and grB expression, while maximal responses were generated by 2C11Rα15 microspheres. Combined stimulation of CD8$^+$ T-cells with 2C11Rα and BSA or Rα microspheres resulted in no augmentation of responses compared to 2C11Rα microspheres alone. Interestingly, the combination of Rα15 and

2C11Rα microspheres led to significantly increased IFN-γ and grB production, as well as enhanced proliferation compared to when either was cultured alone. However, the responses were consistently lower than that induced by 2C11Rα15 microspheres. Taken together, our findings suggest that IL-15 transpresentation can substantially augment TCR stimulation even when presented on a separate surface. Furthermore, to optimally enhance anti-CD3ε mediated responses, transpresented IL-15 should be co-displayed on the same surface.

IL-15 TRANSPRESENTATION ENHANCES EX VIVO LCMV SPECIFIC MEMORY CD8$^+$ T-CELL RESPONSES

Thus far, we have addressed the ability of IL-15 transpresentation to augment anti-CD3ε induced activation of CD8$^+$ T-cells from unimmunized C57BL/6 mice. Physiological CD8$^+$ T-cell activation occurs only upon TCR recognition of cognate peptide antigen presented by class I MHC whereas, anti-CD3ε stimulation results in the polyclonal activation of responding CD8$^+$ T-cells. In addition, unimmunized mice are populated with both naïve and MP CD8$^+$ T-cells specific for self or environmental antigens. Therefore, our preceding results using anti-CD3ε as a TCR stimulus do not distinguish whether the responding cells were naïve or antigen specific memory CD8$^+$ T-cells. To approach this question, particularly the capacity of IL-15 transpresentation to influence antigen specific memory CD8$^+$ T-cells restimulation, various microsphere constructs were prepared with immobilized recombinant class I MHC fusion proteins (H-2D^b/Ig) together with IL-15Rα/Fc. Following immobilization, microspheres with co-immobilized H-2D^b/Ig and IL-15Rα/Fc were pulsed with either LCMV gp33 or control Influenza NP366 peptides in the presence or absence of IL-15. To investigate the stimulatory capacity of the various microspheres on naïve and antigen-specific MP CD8$^+$ T-cells, C57BL/6 mice were infected by *i.p.* injection of LCMV-Armstrong and allowed sufficient time to generate a population of LCMV specific memory CD8$^+$ T-cells. At least forty days post-infection, CD8$^+$ T-cells were negatively enriched from the spleen and LNs of the LCMV immune C57BL/6 mice, labeled with CFSE, and sorted into naive (CD44^{low}) and MP (CD44^{high}) CD8$^+$ T-cell populations. CFSE labeled naive and MP CD8$^+$ T-cells were cultured with the various microsphere constructs and analyzed at the indicated time points for proliferation and expression of intracellular IFN-γ and grB.

Using this approach, we found that CD44^{low} naive phenotype CD8$^+$ T-cells did not undergo proliferation, or express IFN-γ or grB when cultured with any of the microspheres examined (Fig. 3-4). The lack of responsiveness by the CD44^{low} naïve CD8$^+$ T-cells may have been due to the low frequency of gp33 and NP366 specific CD8$^+$ T-cells in the sorted populations. However, since naïve CD8$^+$ T-cells did not respond to IL-15 transpresenting microspheres regardless of the peptide antigen, it suggested that naïve CD8$^+$ T-cells are unresponsive to transpresented IL-15 alone. Our findings with naïve CD8$^+$ T-cells are consistent with the well established concept that primary activation and expansion of naïve CD8$^+$ T-cells requires stimulation through other co-stimulatory receptors in addition to TCR stimulation (42-44).

In contrast to naïve CD44^{low} CD8$^+$ T-cells, analysis of IFN-γ production by CD44^{high} MP CD8$^+$ T-cells revealed differential responsiveness upon culture with the various microspheres. Stimulation of CD44^{high} MP CD8$^+$ T-cells from LCMV immune mice with Influenza NP366 peptide pulsed microspheres (NP366/Rα) induced no detectable level of IFN-γ expression (Fig. 3-4*A*). Preloading of IL-15 onto NP366/Rα microspheres (NP366/Rα15) also had no effect on IFN-γ production. Taken together the data demonstrated that neither non-specific pMHC complexes nor IL-15 transpresentation in the absence of specific peptide antigen can induce IFN-γ expression by CD44^{high} MP CD8$^+$ T-cells. In contrast, when LCMV gp33 peptide was presented by H-2D^b/Ig in the absence of IL-15 (gp33/Rα) a low percentage of IFN-γ producing cells was detectable, confirming the presence of LCMV gp33 specific memory CD8$^+$ T-cells in the CD44^{high} population. However, IL-15 preloading onto gp33/Rα microspheres (gp33/Rα15) greatly augmented

IFN-γ expression by CD44^{high} MP CD8^{+} T-cells in a synergistic manner as suggested by the three fold increase in the percentage of IFN-γ expressing cells.

In addition to IFN-γ, sorted CD8^{+} T-cells cultured with the various microspheres were analyzed for intracellular grB expression (Fig. 3-4*B*). CD44^{high} MP CD8^{+} T-cells stimulated with NP366/Rα microspheres were found to express no grB, whereas a small percentage of CD44^{high} grB positive CD8^{+} T-cells were present following stimulation with NP366/Rα15 microspheres. The ability of NP366/Rα15 to induce grB was in line with our previous results demonstrating that transpresented IL-15 alone can induce a low level of grB expression in the absence of a TCR stimulus (Fig. 3-2 and 3). Antigen specific stimulation of CD44^{high} MP CD8^{+} T-cells with gp33/Rα beads resulted in a low level of grB expression similar to NP366/Rα15 microspheres. However, gp33/Rα15 microsphere stimulation of CD44^{high} MP CD8^{+} T-cells induced production of grB in a significant percentage of cells far beyond that generated by NP366/Rα15 or gp33/Rα microspheres. The percentage of grB expressing cells was comparable to the percentage of IFN-γ producing cells following stimulation with gp33/Rα15 microspheres (Fig. 3-4*A* and *B*). Taken together, these findings suggest synergy between pMHC and transpresented IL-15 in induction of grB by antigen specific memory CD8^{+} T-cells.

Following 48 hrs of culture with the various microsphere constructs, proliferation of the sorted CD8^{+} T-cell subsets was analyzed by CFSE dilution (Fig. 3-4*C*). Consistent with the IFN-γ and grB expression analysis, NP366/Rα microspheres did not induce any cell division following culture. In contrast to IFN-γ, but similar to grB expression, NP366/Rα15 as well as gp33/Rα microspheres induced significant levels of proliferation by CD44^{high} MP CD8^{+} T-cells from LCMV immune mice. However, following stimulation with gp33/Rα15 microspheres, a doubling in the percentage of divided cells was observed such that approximately 50% of the cells had undergone cell division.

Taken together, our results demonstrate that IL-15 transpresentation alone or in combination with pMHC primarily stimulates CD44^{high} MP CD8^{+} T-cell responses.

Multiple antigen specific memory $CD8^+$ T-cell responses to pMHC complexes were substantially augmented by transpresented IL-15 including IFN-γ and grB production, and possibly proliferation. In the cases of IFN-γ and grB production, the dramatic differences in response levels to antigen or IL-15 transpresentation alone compared to when they are combined, suggest that antigen and IL-15 transpresentation synergize in stimulating LCMV specific memory $CD8^+$ T-cells. Furthermore, since $CD44^{low}$ naive $CD8^+$ T-cell responses were not induced or enhanced by transpresented IL-15, it suggests that the responses seen following anti-CD3ε stimulation in figures 3-1 through 3-3 may have been the result of stimulation of the endogenous MP $CD8^+$ T-cells present in the unimmunized mice.

DIFFERENTIAL RESPONSIVENESS OF CENTRAL MEMORY AND EFFECTOR MEMORY $CD8^+$ T-CELLS TO TRANSPRESENTED IL-15

Since $CD44^{high}$ MP $CD8^+$ T-cells can be further divided into T_{CM} and T_{EM} $CD8^+$ T-cell populations based on the expression of CD62L, we next sought to determine if T_{CM} and T_{EM} $CD8^+$ T-cells have differing responses to transpresented IL-15 following pMHC stimulation. Unfortunately, CD62L is rapidly shed from the cell surface following TCR stimulation through cleavage by the TNFα converting enzyme, thereby making analysis of stimulated populations of bulk or $CD44^{high}$ $CD8^+$ T-cells difficult (45-47). Therefore, CFSE labeled $CD8^+$ T-cells from LCMV immune C57BL/6 mice were sorted into naive, T_{CM} or T_{EM} populations based on CD8α, CD44 and CD62L expression to >95% purity (Fig. 3-5*A*). To examine antigen specific responses to pMHC complexes, the sorted $CD8^+$ T-cell populations were stimulated with peptide pulsed microspheres co-displaying $H\text{-}2D^b$/Ig and IL-15Rα/Fc in the absence (NP366/Rα or gp33/Rα) or presence of transpresented IL-15 (NP366/Rα15 or gp33/Rα15). Following culture with microspheres, sorted T-cell populations were analyzed for CFSE dilution, tetramer binding and intracellular grB expression by flow cytometry.

Following 72 hrs of culture, naive CD8$^+$ T-cells did not proliferate in response to any of the microsphere constructs examined (Fig. 3-5*B*). We also found that T_{EM} CD8$^+$ T-cells underwent very little, if any proliferation following stimulation with either NP366/Rα or NP366/Rα15 microspheres (Fig. 3-5*C*). Following antigen specific stimulation with gp33/Rα or gp33/Rα15 microspheres, T_{EM} CD8$^+$ T-cells were induced to undergo several rounds of division. Tetramer analysis of the T_{EM} CD8$^+$ T-cells that proliferated in response to gp33/Rα or gp33/Rα15 stimulation revealed that a similar proportion of T_{EM} CD8$^+$ T-cells stained with the gp33 tetramer after the same number of cell divisions (Fig. 3-5*C*). A significant amount of antigen non-specific division was noted following stimulation with gp33 peptide pulsed microspheres, as evidenced by proliferating CD8$^+$ T-cells that did not stain with either gp33 or control NP366 tetramers. During an active immune response against LCMV *in vivo*, a large percentage of CD8$^+$ T-cells undergo bystander activation and are not specific for LCMV epitopes (48, 49). This may also be the case following stimulation with gp33 peptide pulsed microspheres, whereby a proportion of the proliferating CD8$^+$ T-cells may be specific for environmental antigens or other non-gp33 LCMV epitopes. Regardless, a strong antigen specific response was detected by gp33 tetramer staining in the responding T_{EM} CD8$^+$ T-cell population and was only slightly enhanced by IL-15 transpresentation (e.g., for cells that underwent four or more divisions).

Similar to T_{EM}, sorted T_{CM} CD8$^+$ T-cells also did not proliferate in response to NP366/Rα microspheres (Fig. 3-5*D*). Interestingly, whereas NP366/Rα15 microspheres were unable to induce proliferation of T_{EM} CD8$^+$ T-cells, they did induce proliferation in 22% of T_{CM} CD8$^+$ T-cells (Fig. 3-5*C* and *D*). Tetramer analysis demonstrated that T_{CM} CD8$^+$ T-cells that underwent one to three rounds of cell division when cultured with NP366/Rα15 did not stain positive for either gp33 or NP366 tetramers, suggesting that transpresented IL-15 alone induced antigen independent proliferation of T_{CM} CD8$^+$ T-cells (Fig. 3-5*D*). Upon stimulation of T_{CM} CD8$^+$ T-cells with gp33/Rα microspheres, a low level of proliferation was found which was substantially reduced compared to T_{EM} CD8$^+$

T-cells cultured with the same gp33/Rα microspheres (Fig. 3-5*C* and *D*). In addition, regardless of the division number, a proportion of gp33/Rα stimulated T_{CM} $CD8^+$ T-cells stained positive with the gp33 tetramer. When the T_{CM} $CD8^+$ T-cells were stimulated with gp33/Rα15 microspheres, there was a significant augmentation of proliferation that was equivalent to the proliferative response seen when T_{EM} $CD8^+$ T-cells were stimulated with either gp33/Rα or gp33/Rα15 microspheres (Fig. 3-5*C* and *D*). The dependence of antigen stimulated T_{CM} $CD8^+$ T-cells on transpresented IL-15 to undergo numerous rounds of cell division (e.g., greater than four rounds) was particularly pronounced (Fig. 3-5*D*). Tetramer analysis revealed that T_{CM} $CD8^+$ T-cells that underwent one to three rounds of division in response to gp33/Rα15 stimulation were mostly antigen non-specific, whereas T_{CM} $CD8^+$ T-cells that divided four or more times contained the majority of gp33 tetramer positive cells. Taken together, our results suggest that T_{EM} $CD8^+$ T-cells proliferate more effectively than T_{CM} $CD8^+$ T-cells in response to antigen specific pMHC stimulation alone, and that T_{CM} $CD8^+$ T-cells are more dependent on IL-15 transpresentation than T_{EM} $CD8^+$ T-cells to proliferate following antigen specific stimulation. Furthermore, due to an increased responsiveness to transpresented IL-15, T_{CM} $CD8^+$ T-cells undergo a limited amount of pMHC independent cell division following stimulation with transpresented IL-15 alone, whereas T_{EM} $CD8^+$ T-cells require pMHC stimulation for the initiation of cell division.

In addition to cellular proliferation, grB expression by T_{EM} and T_{CM} $CD8^+$ T-cells was analyzed following 72 hrs of culture (Fig. 3-5*E*). NP366/Rα microspheres did not stimulate grB expression in either memory population, whereas NP366/Rα15 microspheres induced grB expression in both T_{EM} and T_{CM} $CD8^+$ T-cells. Following gp33/Rα stimulation T_{EM}, but not T_{CM} $CD8^+$ T-cells responded by expressing grB. Consistent with our previous findings (Fig. 3-4), gp33/Rα15 stimulation augmented grB expression of both T_{EM} and T_{CM} $CD8^+$ T-cells (Fig. 3-5*E*). Thus, the presence of transpresented IL-15 substantially augments the normally minimal T_{CM} $CD8^+$ T-cell grB response to pMHC, whereas T_{EM} $CD8^+$ T-cells mount an effective grB response to pMHC stimulation alone. Interestingly, IL-15 transpresentation alone could induce grB expression in T_{EM} CD8$^+$ T-cells but was unable to induce antigen independent proliferation (Fig. 3-5*C* and *E*). In contrast, T_{CM} CD8$^+$ T-cells responded by both proliferating and expressing grB following transpresented IL-15 stimulation alone (Fig. 3-5*D* and *E*). Taken together, T_{CM} CD8$^+$ T-cells appear to be more dependent on transpresented IL-15 than T_{EM} CD8$^+$ T-cells for the induction of cellular proliferation and grB expression.

CENTRAL MEMORY CD8$^+$ T-CELLS REQUIRE HOST IL-15Rα EXPRESSION FOR OPTIMAL PROLIFERATIVE RESPONSES IN VIVO.

Our findings thus far suggest that T_{CM} CD8$^+$ T-cells require IL-15 transpresentation for optimal proliferative responses *in vitro*; therefore, we next wanted to determine whether this was also occurring *in vivo* following LCMV infection. To address this issue we generated LCMV specific memory CD8$^+$ T-cells in B6x129 mice by *i.p.* infection with LCMV-Armstrong (Fig. 3-6*A*). Forty days post infection CD8$^+$ T-cells were negatively enriched from the spleen and LNs, labeled with CFSE, and sorted into naive, T_{CM} and T_{EM} populations. Equivalent numbers of each CD8$^+$ T-cell population were then adoptively transferred into naive B6x129 or B6x129 *IL-15R*$\alpha^{-/-}$ mice by *i.v.* injection. Twenty four hrs post transfer, recipient mice were infected with LCMV-Armstrong *i.p.* Four days post-infection spleens and LNs were harvested and CFSE dilution profiles of the adoptively transferred CD8$^+$ T-cell populations were examined by flow cytometry. Since our analysis was limited to CFSE dilution of the adoptively transferred CD8$^+$ T-cells, our results would not be complicated by the detection of host CD8$^+$ T-cell proliferation.

Naive CD8$^+$ T-cell proliferation was negligible in the spleen and LNs of both B6x129 and B6x129 *IL-15R*$\alpha^{-/-}$ mice four days post LCMV infection (Fig. 3-6*B* and *C*). Proliferation of both T_{EM} and T_{CM} CD8$^+$ T-cells following LCMV infection was reduced in B6x129 *IL-15R*$\alpha^{-/-}$ mice compared to B6x129 mice; however, proliferation of T_{CM} CD8$^+$ T-cells was reduced to a much greater extent than T_{EM} $CD8^+$ T-cells. In the spleen there was a 30% reduction in proliferation of T_{EM} in B6x129 *IL-15Rα$^{-/-}$* mice compared to a 65% reduction in proliferation for T_{CM} (Fig. 3-6*B*). There was a similar 36% and 68% reduction in cell division by T_{EM} and T_{CM} $CD8^+$ T-cells respectively in the LNs of B6x129 *IL-15Rα$^{-/-}$* mice compared to B6x129 mice (Fig. 3-6*C*). Importantly, LCMV infection of B6x129 mice demonstrated comparable proliferative capacity of T_{CM} and T_{EM} $CD8^+$ T-cells in both the spleen and LNs. Taken together, our results suggest that both T_{EM} and T_{CM} $CD8^+$ T-cell proliferation is augmented in the presence of IL-15Rα *in vivo* and T_{CM} $CD8^+$ T-cells are more dependent on host IL-15Rα expression for optimal recall responses to LCMV re-exposure *in vivo*.

DISCUSSION

In recent years, significant progress has been made in understanding the role of IL-15 in diverse aspects of $CD8^+$ T-cell function. Following its initial cloning and characterization, several studies demonstrated the ability of IL-15 to induce $CD8^+$ T-cell activation (14, 50). However, these analyses were limited to the expression of early activation markers and secretion of cytokines by unfractionated spleen and PBMC populations (16-19). Furthermore, transpresentation of IL-15 by the high affinity IL-15Rα chain has only been recently discovered and therefore was not taken into account during these early studies (27). Evidence now suggests that IL-15 transpresentation is the primary physiological mechanism of IL-15 function *in vivo* and requires the co-ordinate expression of both IL-15Rα and IL-15 by DCs or a similar hematopoietic cell type (24). Recent studies have also demonstrated that *in vivo* administration of soluble IL-15Rα/Fc complexed with IL-15 is able to induce hyper-agonistic proliferation of memory $CD8^+$ T-cells (28, 51, 52). The proliferation seen in these studies was in the complete absence of TCR stimulation and required extremely high non-physiological serum concentrations of IL-15. Thus, in spite of the vast amount of studies conducted on this intriguing cytokine, there has been limited data regarding the contribution of IL-15 transpresentation to TCR induced activation of $CD8^+$ T-cells. Our approach using microspheres allowed us to specifically examine the role of IL-15 transpresentation in augmenting TCR induced activation of $CD8^+$ T-cells, in the absence of other stimulatory signals. Ligand immobilization on microspheres as single ligands, or combinations can be performed to result in physiological ligand densities similar to those observed on DCs. Therefore, microspheres offered the ideal platform to specifically address the role of IL-15 transpresentation on TCR stimulation induced $CD8^+$ T-cell activation.

In this report, we have demonstrated that transpresented IL-15 is significantly more effective than soluble IL-15 at equivalent concentrations in augmenting anti-CD3ε induced $CD8^+$ T-cell proliferation and effector molecule expression. It remains to be determined whether transpresented IL-15 is more effective than soluble IL-15 at augmenting antigen specific $CD8^+$ T-cell responses; however, in this study our emphasis was on IL-15 transpresentation since it appears to be the primary mechanism of IL-15 action *in vivo* (23-26). Furthermore, IL-15Rα deficient mice are capable of producing IL-15 therefore, if soluble IL-15 was as effective as transpresented IL-15 in augmenting antigen specific responses we may not have seen such dramatic reductions in proliferation of responding $CD8^+$ T-cells in IL-15Rα deficient mice that are incapable of transpresenting IL-15. However, it would be interesting to examine antigen specific responses in mice deficient in both IL-15Rα and IL-15 to determine the contribution of soluble IL-15 to antigen specific recall responses. Nevertheless, our results demonstrate that transpresentation is significantly more effective than soluble IL-15 at augmenting anti-CD3ε induced $CD8^+$ T-cell activation and upon titration of the density of transpresented IL-15, a clear dose dependent augmentation of anti-CD3ε induced activation was evident.

Further examination of the role of IL-15 transpresentation in $CD8^+$ T-cell responses revealed that transpresented IL-15 synergizes with TCR stimulation to augment CD8$^+$ T-cell responses. The synergism was particularly apparent following CD8$^+$ T-cell stimulation with anti-CD3ε and transpresented IL-15 whereby the combined effect of the two stimuli was far beyond the additive effects of the individual stimuli for each of the responses examined. Upon pMHC stimulation of CD44^{high} MP CD8$^+$ T-cells, the synergistic relationship applied only to IFN-γ and grB expression, whereas the combined effect on proliferation appeared to be additive rather than synergistic. However, when CD44^{high} MP CD8$^+$ T-cells were further divided into T_{CM} and T_{EM} populations, it was apparent that pMHC and IL-15 transpresentation synergize to enhance proliferation of antigen specific T_{CM} CD8$^+$ T-cells. In contrast, the proliferative response of antigen specific T_{EM} CD8$^+$ T-cells was not significantly enhanced by transpresented IL-15 following pMHC stimulation *in vitro*. Taken together, our findings indicate that IL-15 transpresentation augments TCR stimulated MP and antigen specific memory CD8$^+$ T-cell activation, in a synergistic manner. Finally, our observation that naïve CD44^{low} CD8$^+$ T-cells were unresponsive to transpresented IL-15 suggests that transpresented IL-15 may not play a major role during the primary activation of naïve CD8$^+$ T-cells, however our experiments have not formally ruled out this possibility. Interestingly, IL-15 and anti-CD3ε have been shown to induce highly similar gene expression patterns in MP CD8$^+$ T-cells however, the combined effects of IL-15 and anti-CD3ε on gene expression was not examined (20). It would therefore be interesting to determine whether combined IL-15 and anti-CD3ε or pMHC stimulation alters the expression pattern of genes not affected by either stimulus alone, or whether there is a corresponding enhancement or reduction in the shared gene transcripts.

Our demonstration that IL-15 transpresentation could augment anti-CD3ε immobilized on a separate bead surface suggests several interesting possibilities. Our results showed that transpresented IL-15 alone resulted in a low level of proliferation and grB expression by MP CD8$^+$ T-cells but not naïve CD8$^+$ T-cells. Serial encounter of CD8$^+$ T-cells with activated DCs would likely result in more frequent interactions with transpresented IL-15 rather than MHC presenting a specific antigenic peptide.

Therefore, transpresented IL-15 may provide a low level of stimulation to responding MP CD8$^+$ T-cells and upon TCR stimulation, MP CD8$^+$ T-cells would be poised to mount a robust recall response. IL-15 transpresentation may therefore serve a dual purpose by providing necessary survival signals and prepare MP CD8$^+$ T-cells for enhanced antigen specific activation. This may especially be the case for T_{CM} CD8$^+$ T-cells that are highly responsive to transpresented IL-15 and are also enriched in LNs where they would regularly interact with activated DCs. A second intriguing possibility is that activated DCs transpresenting IL-15 may provide signals to nearby MP CD8$^+$ T-cells receiving TCR stimulation from a separate DC. This situation would require a CD8$^+$ T-cell to maintain multiple contacts with separate DCs, which could occur during DC and T-cell aggregation (53). Since IL-15 transpresentation can augment TCR stimulation provided on a separate surface, the synergism between IL-15 transpresentation and TCR stimulation presented on the same surface or cell could not simply be due to increased adhesion resulting in enhanced or prolonged interactions with a TCR ligand. If this were the case, IL-15 transpresentation could only enhance responses when co-presented with a TCR stimulus. Taken together, the ability of IL-15 transpresentation to act as an independent stimulator of MP CD8$^+$ T-cell responses and also function in combination with TCR stimulation suggests that IL-15 plays a complex role in CD8$^+$ T-cell immune function.

Based on tissue localization, functional characteristics and cell surface markers, memory T-cells have been broadly divided into T_{CM} and T_{EM} populations (54, 55). We have shown in this report that T_{CM} and T_{EM} CD8$^+$ T-cells have differential responsiveness to transpresented IL-15. T_{CM} CD8$^+$ T-cells undergo limited cell division following stimulation with transpresented IL-15 alone, whereas T_{EM} CD8$^+$ T-cells are relatively unresponsive to transpresented IL-15 in the absence of antigen. In our hands, T_{EM} and T_{CM} CD8$^+$ T-cells have similar proliferative capacities following optimal stimulation, so the lack of T_{EM} CD8$^+$ T-cell proliferation following transpresented IL-15 stimulation alone does not correlate with an intrinsic lower proliferative ability. Furthermore, similar to T_{CM} CD8$^+$ T-cells, T_{EM} CD8$^+$ T-cells can be induced to express grB following transpresented IL-15 stimulation alone. Taken together, these results suggest that T_{CM} and T_{EM} $CD8^+$ T-cells have different responsiveness to transpresented IL-15. In regards to TCR stimulation, T_{EM} $CD8^+$ T-cells vigorously proliferate and express grB following TCR stimulation alone, whereas T_{CM} $CD8^+$ T-cells have negligible responses following antigen pMHC stimulation alone. However, if transpresented IL-15 is provided together with antigen pMHC stimulation, T_{CM} $CD8^+$ T-cell responses are equivalent to the responses mediated by T_{EM} $CD8^+$ T-cells. In striking contrast, T_{EM} $CD8^+$ T-cell responses were only slightly enhanced when transpresented IL-15 was provided together with antigen pMHC stimulation. These findings were further supported by our *in vivo* adoptive transfer experiments. T_{CM} $CD8^+$ T-cells from LCMV immune mice adoptively transferred into IL-15Rα deficient mice had minimal proliferative responses following LCMV challenge; in contrast, T_{EM} $CD8^+$ T-cell proliferation was reduced but not absent in IL-15Rα deficient mice. Taken together, our results suggest that T_{EM} $CD8^+$ T-cells require only cognate pMHC stimulation to initiate recall responses to a previously encountered pathogen, whereas T_{CM} $CD8^+$ T-cells are more dependent on transpresented IL-15 for optimal responses both *in vitro* and *in vivo*. It has been suggested that since T_{EM} $CD8^+$ T-cells are prevalent in non-lymphoid tissue, they may require less stringent activation criteria to facilitate rapid responses upon antigen re-exposure, whereas T_{CM} $CD8^+$ T-cells that reside primarily in lymphoid tissues may require interaction with DCs to initiate their full activation. In support of this concept, *in vivo* DC depletion experiments have revealed that T_{CM} $CD8^+$ T-cells are more dependent on DCs than T_{EM} $CD8^+$ T-cells following vesicular stomatitis virus infection, although both mounted equivalent responses in the presence of DCs (31). Taken together, our findings may provide a clue as to how the division of labor between T_{CM} and T_{EM} $CD8^+$ T-cells may be controlled by differential activation requirements. Therefore, in addition to differences attributable to tissue localization, activation requirements may play an important role in determining the contribution of T_{CM} and T_{EM} $CD8^+$ T-cells to secondary recall responses upon pathogen re-encounter. T_{EM} $CD8^+$ T-cells may provide a first line of defense since they can respond in a rapid manner due to their reduced activation requirements (e.g., a TCR stimulus in the absence of IL-15 transpresentation) and localization within peripheral tissues; whereas, T_{CM} $CD8^+$ T-cell activation requires additional signals provided by activated DCs. Type I IFNs and TLR ligands are required for the co-ordinate upregulation of IL-15 and IL-15Rα by DCs and may therefore regulate T_{CM} $CD8^+$ T-cell responses such that they occur only during times of inflammation (24-26, 29). Migration of activated DCs transpresenting IL-15 together with an appropriate TCR ligand could function to regulate recall responses by activating antigen specific T_{CM} $CD8^+$ T-cells only during situations when T_{EM} $CD8^+$ T-cell responses are unable to control the infection.

Finally, our findings may have significant implications for the use of artificial cell surfaces for the propagation of memory $CD8^+$ T-cells for immunotherapy (56-58). Our results clearly define a role of transpresented IL-15 in the reactivation of memory $CD8^+$ T-cells, especially for T_{CM} $CD8^+$ T-cells. Therefore, IL-15 transpresentation may provide utility in immunotherapeutic strategies aimed at enhancing antigen specific memory $CD8^+$ T-cell reactivation, particularly *ex vivo* expansion of memory $CD8^+$ T-cells for adoptive immunotherapeutic approaches.

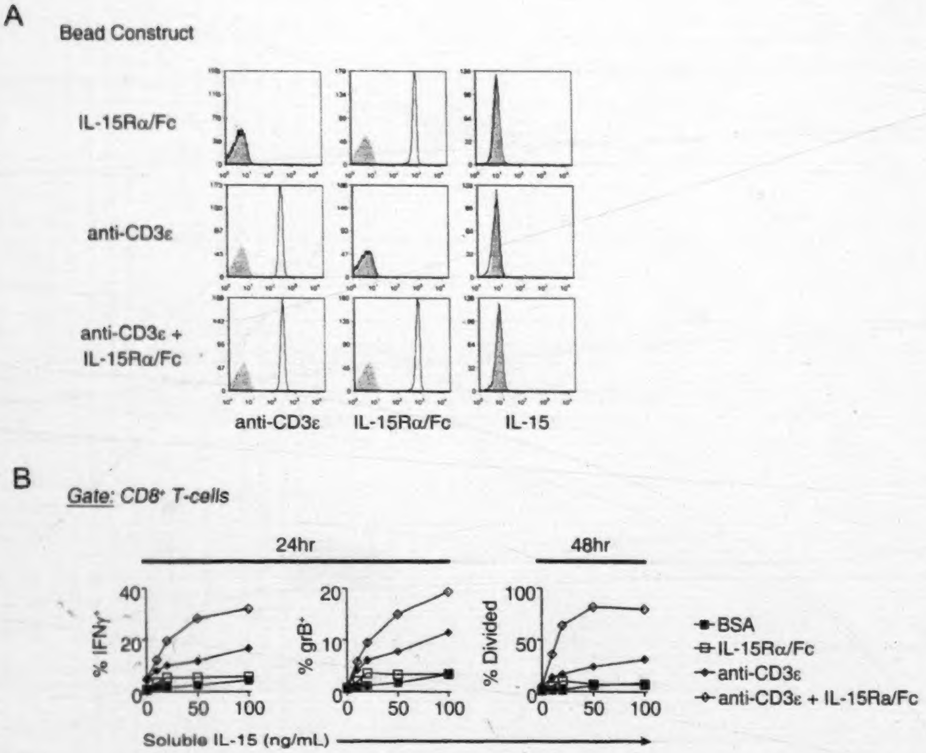

Figure 3-1. Soluble IL-15 is more effective at augmenting anti-CD3ε induced CD8$^+$ T-cell stimulation in the presence of IL-15Rα/Fc. A) Immobilization of anti-CD3ε and IL-15Rα/Fc onto cell-sized microspheres. FACS histograms of microsphere constructs with immobilized anti-CD3ε, IL-15Rα/Fc, or both. Immobilized anti-CD3ε was detected by a FITC-conjugated goat anti-hamster IgG. IL-15Rα/Fc and IL-15 were detected using polyclonal goat anti-IL-15Rα or IL-15, followed by an R-PE-conjugated donkey anti-goat IgG F(ab')$_2$. Shaded histograms represent staining of BSA microspheres with the indicated antibodies. B) Soluble IL-15 in the presence of immobilized IL-15Rα/Fc augments anti-CD3ε activation of CD8$^+$ T-cells. Negatively enriched, CFSE labeled C57BL/6 CD8$^+$ T-cells were cultured with the indicated microsphere constructs in thepresence of soluble IL-15 at a final concentration of 0 – 100ng/mL. At the indicated time points, cells were analyzed for intracellular IFN-γ and grB and CFSE dilution by flow cytometry. CD8$^+$ T-cells were pooled from multiple mice; therefore, sample size is 1. Data represent means of triplicate samples ± SEM. 30,000 gated events were acquired per sample.

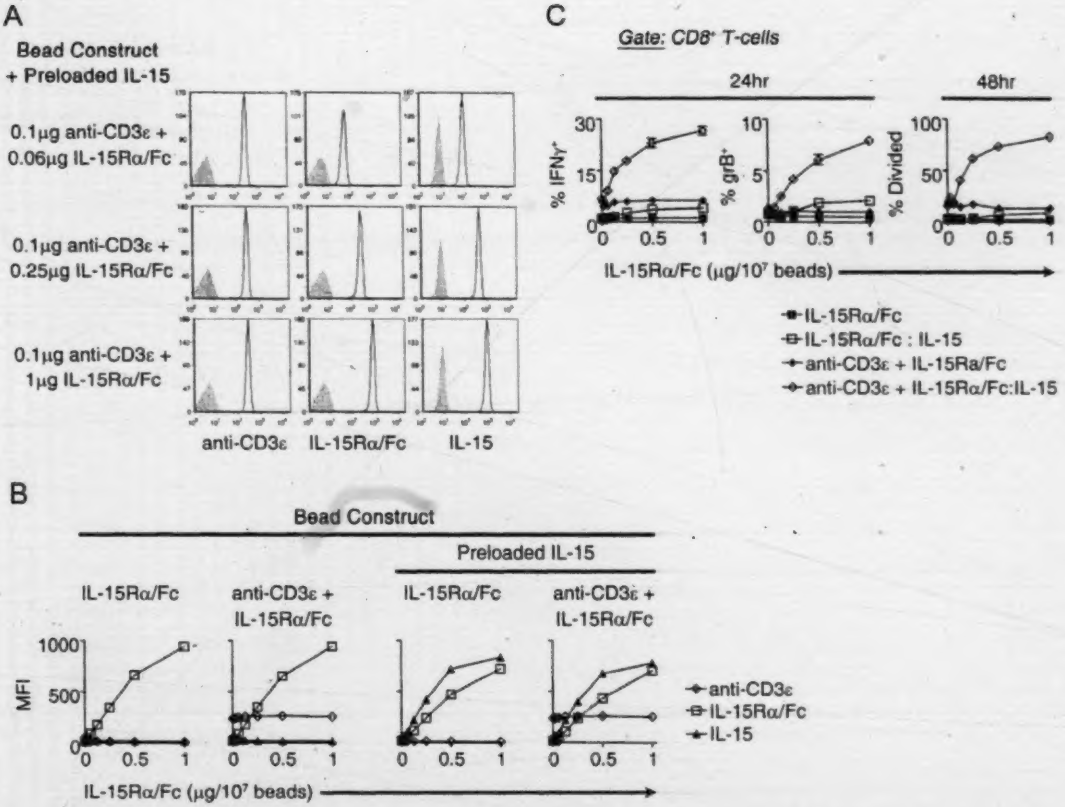

Figure 3-2. Co-immobilized anti-CD3ε and transpresented IL-15 synergize in stimulating CD8$^+$ T-cell responses. A) Titration of immobilized IL-15Rα/Fc and IL-15 on cell sized microspheres. FACS histograms of microsphere constructs with co-immobilized anti-CD3ε and various amounts of IL-15Rα/Fc preloaded with IL-15. 1x10^7 of the various microsphere constructs were incubated overnight in the presence of 100ng of IL-15 at 4°C with rotation. Following incubation, microspheres were washed extensively to remove any unbound IL-15 and resuspended in culture medium. Microsphere constructs were stained as previously described, in *Materials and Methods*. Shaded histograms represent staining of BSA microspheres with the indicated antibodies. B) The MFI staining of immobilized anti-CD3ε, IL-15Rα/Fc and IL-15, plotted against the amount of IL-15Rα/Fc immobilized on microspheres. Partially reduced staining of IL-15Rα/Fc seen in the presence of IL-15 is likely due to the polyclonal nature of the IL-15Rα detection antibody and the loss of some antibody binding sites following the binding of IL-15. Density of immobilized anti-CD3ε is maintained constant while the density of IL-15Rα/Fc can be precisely adjusted with a corresponding change in the amount of transpresented IL-15. C) Dose dependent augmentation of anti-CD3ε induced CD8$^+$ T-cell activation by transpresented IL-15. Negatively enriched, CFSE labeled C57BL/6 CD8$^+$ T-cells were cultured with various microsphere constructs for 24 and 48 hrs. At the indicated time points, cells were harvested and analyzed for intracellular IFN-γ and grB, or CFSE dilution by flow cytometry. CD8$^+$ T-cells were pooled from multiple mice; therefore, sample size is 1. Data represent means of triplicate samples ± SEM. 30,000 gated events were acquired per sample.

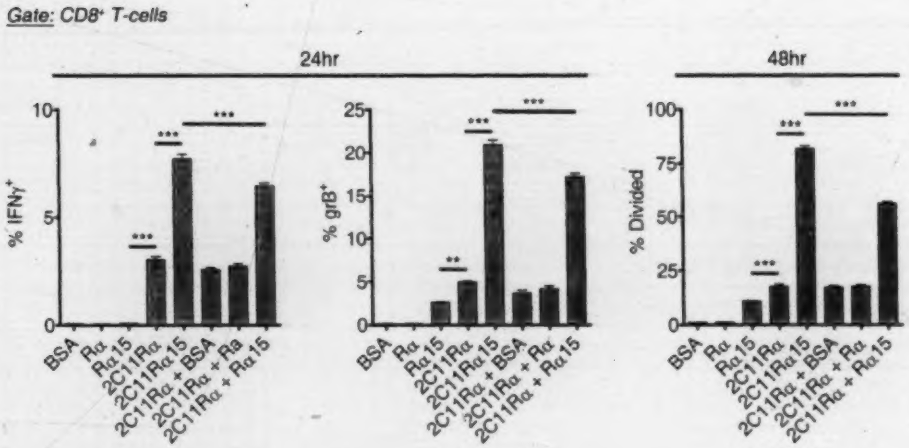

Figure 3-3. CD8$^+$ T-cell responses are augmented when transpresented IL-15 and anti-CD3ε are presented on separate surfaces, and co-presentation of transpresented IL-15 and anti-CD3ε provides optimal CD8$^+$ T-cell stimulation. Negatively enriched, CFSE labeled C57BL/6 CD8$^+$ T-cells were cultured with various microsphere constructs alone or in combination for 24 and 48 hrs. Two hundred and fifty thousand CD8$^+$ T-cells were cultured with 0.5x10^6 of each indicated microsphere construct (total 0.5 - 1x10^6 microspheres). The various microsphere constructs used are abbreviated as follows: IL-15Rα/Fc (Rα), IL-15Rα/Fc:IL-15 (Rα15), 145-2C11 + IL-15Rα/Fc (2C11Rα), 145-2C11 + IL-15Rα/Fc:IL-15 (2C11Rα15), "+" indicates the combination of two different microsphere constructs in the culture (e.g. 2C11Rα microspheres + BSA microspheres (2C11Rα + BSA)). At 24 and 48 hrs of culture, the cells were harvested and analyzed by flow cytometry for intracellular IFN-γ and grB, and for CFSE dilution. CD8$^+$ T-cells were pooled from multiple mice; therefore, sample size is 1. Data represent means of triplicate samples ± SEM. >40,000 gated events were acquired per sample. One-way ANOVA analysis with Bonferroni's multiple comparison test performed with Graphpad Prism software (*** = p<0.001, ** = P<0.01).

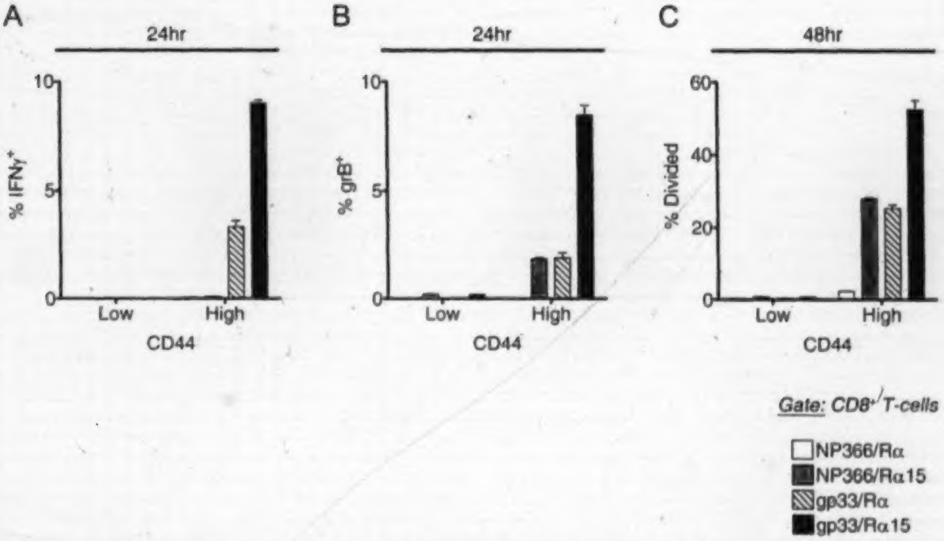

Figure 3-4. IL-15 transpresentation enhances *ex vivo* LCMV specific memory CD8$^+$ T-cell responses. CD8$^+$ T-cells were negatively enriched from the spleen and LNs of C57BL/6 mice previously infected *i.p* with LCMV-Armstrong (>40 days post-infection). CD8$^+$ T-cells were labeled with CFSE, stained for CD8α and CD44, and subsequently sorted for CFSE$^+$ CD8α$^+$ CD44^{low} and CFSE$^+$ CD8α$^+$ CD44^{high} populations. Sorted CD8$^+$ T-cell populations were cultured with NP366/Rα, NP366/Rα15, gp33/Rα or gp33/Rα15 microsphere constructs for 24 and 48hrs. Cells were harvested and analyzed by flow cytometry for intracellular A) IFN-γ, B) grB and C) CFSE dilution. CD8$^+$ T-cells were pooled from multiple mice and sorted into CD44 low and CD44 high populations; therefore, sample size is 1. Data represent means of triplicate samples ± SEM. >30,000 gated events were acquired per sample.

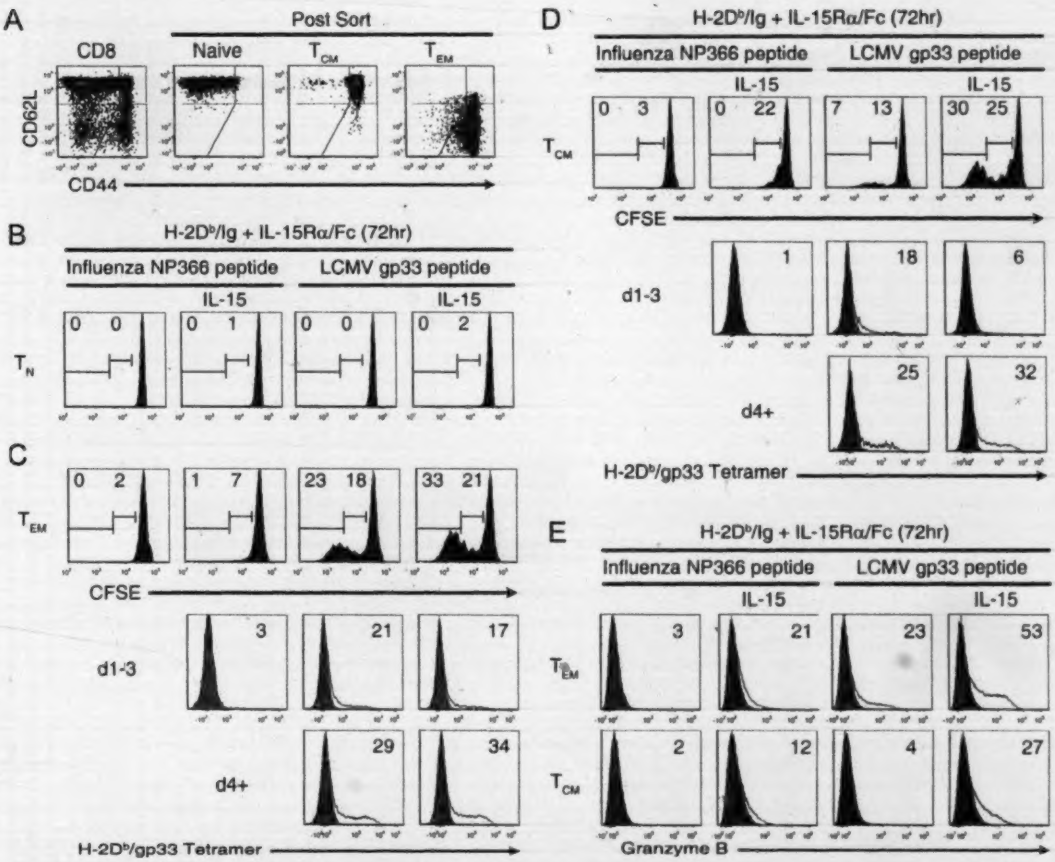

Figure 3-5. Differential responsiveness of T_{CM} and T_{EM} CD8$^+$ T-cells to transpresented IL-15 in the presence and/or absence of antigen stimulation. CD8$^+$ T-cells were negatively enriched from the pooled spleens of C57BL/6 mice previously infected with LCMV-Armstrong (>40 days post-infection). A) Enriched CD8$^+$ T-cells were labeled with CFSE, stained for CD8α, CD44 and CD62L, and subsequently sorted by flow cytometry into naïve, T_{CM}, and T_{EM} populations. Purified B) T_N, C) T_{EM} and D) T_{CM} CD8$^+$ T-cells were cultured with NP366/Rα, NP366/Rα15, gp33/Rα, or gp33/Rα15 microspheres for 72 hrs. Microsphere-stimulated CD8$^+$ T-cell populations were harvested and stained with H-2D^b/gp33 or H-2D^b/NP366 tetramers and mAbs against CD8α, CD62L, and CD44. CFSE dilution and tetramer staining was analyzed by flow cytometry. CFSE division markers represent cells that have undergone 1-3 divisions and 4+ divisions, right to left. Percent of divided cells within each division group is indicated above each marker gate in upper panels. In middle and lower panels of C) and D), open histograms represent staining with H-2D^b/gp33 tetramers, shaded histograms represent staining with H-2D^b/NP366 tetramers. One representative experiment of three is shown. E) Histograms represent intracellular grB staining of the T_{EM} and T_{CM} CD8$^+$ T-cell populations cultured with the microsphere constructs described in C) and D). Open histograms correspond to staining with anti-grB, shaded histograms represent staining with a mouse IgG1 isotype control. Overton subtraction was used to calculate percent H-2D^b/gp33 tetramer and grB positive populations. For all flow cytometric analysis, >30,000 CD8$^+$ T-cell events were acquired. One representative experiment of three shown, n=1.

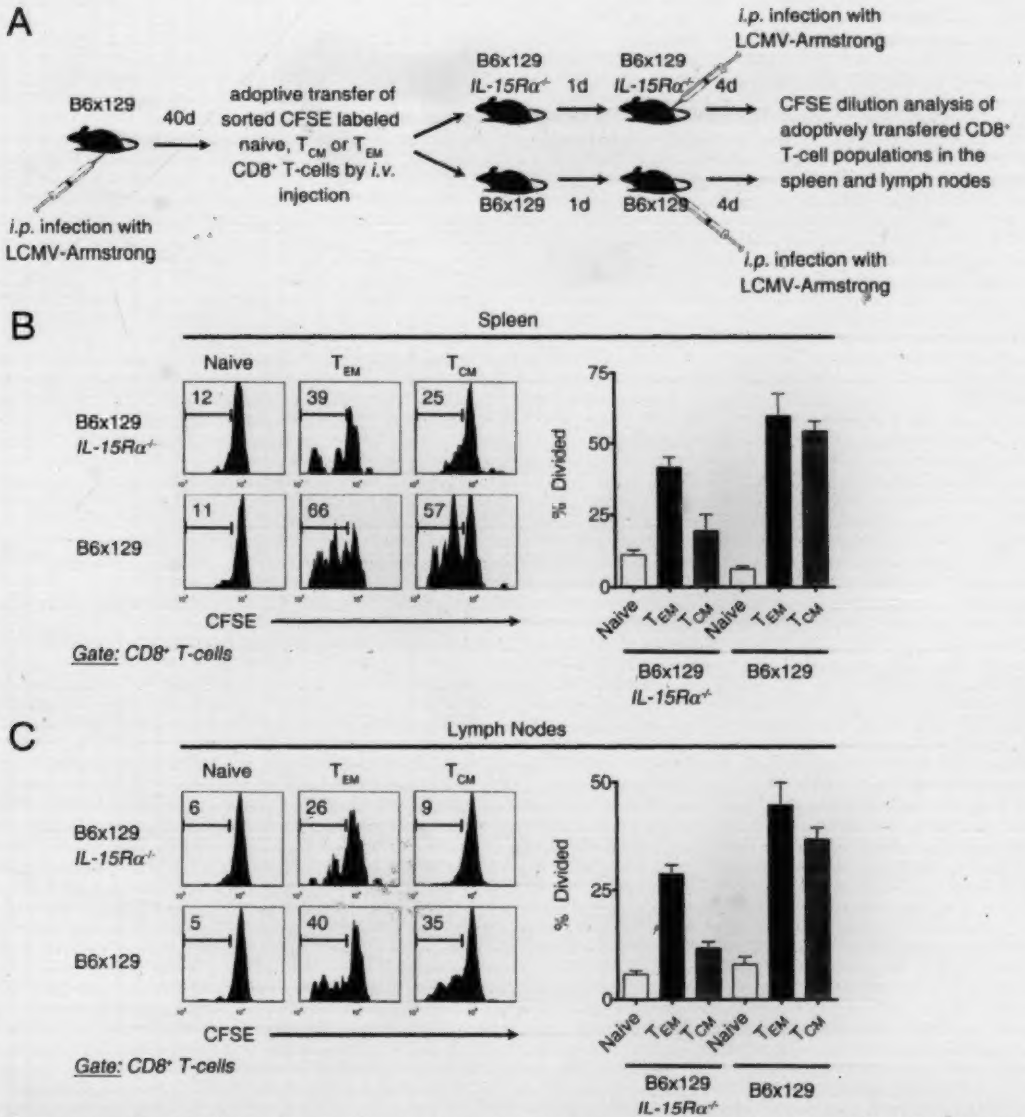

Figure 3-6. T_{CM} CD8⁺ T-cells require host IL-15Rα expression for optimal proliferation in vivo following LCMV infection. A) CD8⁺ T-cells were negatively enriched from pooled spleens from B6x129 mice previously infected with LCMV-Armstrong (>40 days post-infection) and labeled with CFSE and stained for CD8α, CD44 and CD62L. Stained CD8⁺ T-cells were subsequently sorted for naïve, T_{CM}, and T_{EM} populations by flow cytometry. Equivalent numbers of sorted CD8⁺ T-cell populations were adoptively transferred by *i.v.* injection into B6x129 and B6x129 *IL-15Rα*$^{-/-}$ mice. The following day, recipient mice were infected by *i.p.* injection of $2x10^5$ PFU LCMV-Armstrong. Four days post infection, LNs and spleens were harvested and analyzed by flow cytometry. Histograms are CFSE dilution profiles of the adoptively transferred naïve, T_{CM}, and T_{EM} CD8⁺ T-cells in the B) spleen and C) LNs following LCMV infection. Bar graphs represent percent divided of each adoptively transferred population from three mice ± SEM, n=1.

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CHAPTER 4

HIGH LY-6C ISOFORM EXPRESSION UNIQUELY IDENTIFIES RESTING MEMORY CD8$^+$ T-CELLS IN SECONDARY LYMPHOID ORGANS

INTRODUCTION

Molecules of the Ly-6 superfamily have been extensively used as cell surface markers of hematopoeitic stem cells, lymphoid precursors and lymphocytes (1). These small 12-14 kDa GPI-anchored Ly-6 membrane proteins are encoded by approximately twenty linked genes at band E1 of chromosome 15 (1). Multiple Ly-6 family members have been described, including Ly-6A/E (Sca-1), C, D/ThB, F, G (Gr-1), H, I, K, M and TSA-1 (Sca-2) (1-5). Although the ligands of Ly-6 molecules are largely unknown, numerous diverse functions have been proposed for these cell surface molecules, ranging from signal transduction to intercellular adhesion. For instance, Ly-6C has been demonstrated to function as an adhesion molecule for splenic macrophage progenitors and endothelial cells (6, 7). More commonly, due to their wide and unique tissue distribution patterns, Ly-6 molecules are used as differential markers for hematopoeitic cells. As examples, Sca-1 and Ly-6G are used as markers to identify hematopoeitic stem cells and BM granulocytes, respectively (2, 8).

With respect to memory T-cells, Walunas *et al.* were the first to describe a correlation between Ly-6C expression and memory CD8$^+$ T-cells (9). They showed that Ly-6C is permanently up-regulated on antigen experienced CD8$^+$ T-cell populations following anti-CD3ε treatment or tumor challenge *in vivo*, but the use of Ly-6C alone as a marker to isolate antigen specific memory T-cells has not been demonstrated (9). In addition, the kinetics of Ly-6C expression during the natural course of infection leading to the generation of Ly-6C$^+$ memory CD8$^+$ T-cells is also not known. Nevertheless, in conjunction with CD44, CD45, CD62L, CD122 and CD127 (IL-7Rα), Ly-6C has frequently been used as marker to identify memory CD8$^+$ T-cells (9, 10). However, a fraction of CD8$^+$ T-cells isolated from antigen inexperienced animals are also Ly-6C$^+$ and express this GPI-linked molecule to varying extents, and recently, it was shown that the gut microenvironment supports a distinct population of memory T-cells which down-regulated Ly-6C expression (6, 11). These observations raise the question of what the relationship is between Ly-6C expression and memory T-cells. To complicate the issue, Ly-6C exhibits microheterogeneity in that not all Ly-6C specific mAbs show the same reactivity toward Ly-6C (12). Therefore, it is important to re-evaluate the relationship of Ly-6C expression with CD8$^+$ memory T-cells using additional anti-Ly-6C mAbs.

In this chapter, a mAb named iMap is described that recognizes a unique form of the Ly-6C molecule. The iMap reactive Ly-6C (referred to as Ly-6C^{iMap} hereafter) demonstrated an overlapping expression pattern with Ly-6C detected by the anti-Ly-6C mAb, AL-21 (Ly-6C^{AL-21}). Both Ly-6C molecules are expressed by a variety of hematopoeitic cells, but unlike Ly-6C^{AL-21}, Ly-6C^{iMap} is not expressed by resting CD4$^+$ T-cells. Although such differential reactivity has been reported for other anti-Ly-6C antibodies, the iMap reactivity only partially overlapped with these antibodies. Analysis of adult resting T-cells indicated that only CD8$^+$ T-cells expressing a high level of Ly-6C^{iMap} exhibited a memory phenotype. This observation was corroborated by the *in vivo* LCMV infection model, whereby Ly-6C^{iMap} was rapidly up-regulated on activated CD4$^+$ and CD8$^+$ T-cells upon viral infection. Once the infection was resolved, Ly-6C^{iMap} expression returned to a background level; however, a small pool of antigen specific Ly-6C$^{iMap(hi)}$ CD8$^+$ T-cells persisted well into the memory phase of the primary response. Analysis of the CD8$^+$ T-cells isolated from LCMV-immune mice showed that only Ly-6C$^{iMap(hi)}$ CD8$^+$ T-cells produced IFN-γ upon antigen stimulation, confirming that not all Ly-6C$^+$ CD8$^+$ T-cells are memory T-cells. Currently no single marker exists for the identification and isolation of functional memory CD8$^+$ T-cells. We show here that the

Ly-6C$^{iMap(hi)}$ phenotype alone is sufficient to isolate memory CD8$^+$ T-cells from secondary lymphoid organs that can confer protection against LCMV challenge following adoptive transfer into naïve mice.

MATERIALS AND METHODS

MICE

Eight to twelve week old mice were purchased from Charles River Laboratories (Kingston, ON, Canada), Jackson Laboratory (Bar Harbor, ME) and the University of Alberta mouse breeding facility (Edmonton, Alberta, Canada). All animal studies followed the guidelines of the Canadian Council on Animal Care and the University of Alberta Health Animal Policy and Welfare Committee.

CELL LINES

All cell lines were maintained in DMEM or RPMI-1640 supplemented with 5% FBS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Invitrogen, Burlington, ON, Canada). The MDAY-D2 lymphoma was obtained from Dr. J.W. Dennis (Samuel Lunenfeld Research Institute, Toronto, ON, Canada). RF33.70 was kindly provided by Dr. K.L. Rock (University of Massachusetts, Worchester MA). The Ly-6C^{iMap+} RF+ subline was selected by incubating the RF33.70 parental line with the iMap mAb. The Ly-6C^{iMap+} cells were then isolated using anti-IgM conjugated paramagnetic Dynalbeads (Invitrogen). The EL4J Ly-6 transfectants were provided by Dr. T.R. Malek (University of Miami School of Medicine, FL). EL4 and HEK293T were obtained from the American Type Tissue Culture Collection (ATCC, Manassas, VA).

GENERATION OF ADHERENT LYMPHOKINE-ACTIVATED KILLERS

Erythrocyte depleted spleen cells were passaged through nylon wool columns to obtain the nylon wool non-adherent cells. These unbound cells were then cultured in complete RPMI-1640 culture medium supplemented with 800 units/mL of human rIL-2.

MONOCLONAL ANTIBODIES, TETRAMERS AND FLOW CYTOMETRIC ANALYSIS

The iMap (mouse IgM) B-cell hybridoma was generated by immunizing a BALB/c mouse with C57BL/6 adherent lymphokine activated killer cells (A-LAKs), as described (13). Purified iMap was isolated from protein free hybridoma media II (Invitrogen) by $(NH_4)_2SO_4$ precipitation and dialyzed against PBS. B27M2, anti-HLA-B27; and 145-2C11, anti-CD3ε hybridomas were obtained from the ATCC. VL4, anti-LCMV was a gift from Dr. Pamela Ohashi (University of Toronto, ON, Canada). The unconjugated, biotinylated, or fluorochrome-conjugated forms of the following mAbs; 145-2C11, anti-CD3ε; D7, anti-Ly6A/E; AL-21, anti-Ly-6C; RB6-8C5, anti-Ly6G; DX5, anti-CD49b; GK1.5 and L3T4, anti-CD4; 53-6.7, anti-CD8α; IM7, anti-CD44; MEL-14, anti-CD62L; RF70, anti-CD70; MR1, anti-CD40L; H1.2F3, anti-CD69; MB19-1, anti-CD19; PK136, anti-NK1.1; and AN-18, R4-6A2 and XMG1.2, anti-IFN-γ were purchased from eBioscience (San Diego, CA) or BD Biosciences (Mississauga, ON, Canada). Alexa 488 conjugated iMap was prepared using an Alexa 488 protein labeling kit (Invitrogen). The $F(ab')_2$ fragment of PE-conjugated and Cy5-conjugated goat anti-mouse/rat IgM (μ chain-specific) were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA). Fluorochrome-conjugated streptavidin was purchased from Invitrogen. The $H\text{-}2D^b$-gp33 tetramer was obtained from the CANVAC core facilities (Montréal, QC, Canada). To examine iMap epitope expression, one million cells were incubated with 2-5 μg of iMap for 30 min at 4°C. Samples were then counter stained with 100 μl of a 1:200 dilution of Cy5- or PE-conjugated goat anti-mouse/rat IgM(μ chain)-specific $F(ab')_2$ fragments. Upon further washing, cells were fixed in 4% paraformaldehyde in PBS, pH7.1 and analyzed by flow cytometry. Fluorochrome conjugated mAb and tetramer staining was performed at 4°C for 30 mins, followed by washing and fixation as described. Flow cytometric acquisition was performed using a BD FACScan, FACSCanto or FACSAria. Flow cytometric analysis was conducting using BD CellQuest, BD FACSDiva or FCS Express software (DeNovo Software, Thornhill, ON, Canada). Cell sorting was performed on a BD FACSAria.

TRANSFECTION OF HEK293T TO EXPRESS LY-6 MOLECULES

HEK293T cells were transfected with plasmids encoding Ly-6A.2/E.1, Ly-6C.2 or Ly-6I.2 (provided by Dr. A.L. Bothwell, Yale, New Haven, CT) using lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Expression of Ly-6 molecules on transfected HEK293T was assessed by flow cytometry.

ISOLATION OF EX VIVO HEMATOPOEITIC CELLS

Axillary, inguinal, and brachial lymph nodes (LN) and spleens were isolated from C57BL/6 mice and gently disrupted with a tissue homogenizer. Bone marrow cells were isolated from femurs and depleted of erythrocytes with 0.15M NH_4Cl. Total T-cells or $CD8^+$ T-cells were isolated using EasySep enrichment kits (StemCell Technologies Inc., Vancouver, BC, Canada). One-way mixed leukocyte reactions were prepared by culturing C57BL/6 spleen cells with γ-irradiated BALB/c spleen cells for 5 days at a 1:1 ratio. In some instances, splenic T-cells were initially stained and sorted by flow cytometry into Ly-$6C^{iMap+}$ and Ly-$6C^{iMap-}$ populations prior to stimulation with allogeneic spleen cells. The alloreactive C57BL/6 T-cells were maintained by weekly stimulation with γ-irradiated BALB/c spleen cells and human rIL-2.

IN VITRO IL-15 INDUCED DIFFERENTIATION OF MEMORY T-CELLS

C57BL/6 splenic T-cells at $1x10^6$ cells/mL were cultured with 10 ng/mL recombinant mouse IL-15 (Peprotech Inc., Rocky Hill, NJ) for 7 days. The differentiated T-cells were then harvested, stained and analyzed by flow cytometry.

LCMV INFECTIONS

Female C57Bl/6 mice housed in a biocontainment facility were each infected *i.p.* with 2x10^5 PFU of LCMV-Armstrong (gift from Dr. Pamela Ohashi). At indicated time points, BM cells, thymocytes, and splenic and LN T-cells were isolated from the infected mice using an EasySep T-cell enrichment kit and analyzed for Ly-6C expression.

IFN-γ ELISPOT AND FLOW CYTOMETRY BASED IFN-γ ASSAY

Splenic CD8$^+$ T-cells were isolated from C57BL/6 mice infected with LCMV for >40 days and sorted into Ly-6C$^{\text{iMap(neg, lo, int or hi)}}$ populations. EL4 target cells were pulsed with either LCMV peptides (gp33-41, gp276-286, NP396-404 from NeoMPS Inc., San Diego, CA) or influenza A/PR8 peptide (NP366-374, from BIOpeptide Co., San Diego, CA) for 1 hr at 37°C and washed extensively prior to use. Sorted CD8$^+$ T-cells were incubated with peptide pulsed EL4 target cells at a 1:100 E:T ratio in a ninety-six-well MultiScreen-HA plate (Millipore, Bedford, MA) coated with anti-mouse IFN-γ (mAb: AN-18) for 5 hrs at 37°C. At the end of the incubation period the ELISPOT plate was washed and biotin-conjugated anti-mouse IFN-γ (mAb: R4-6A2) was used to detect captured IFN-γ, followed by HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratory). Plates were subsequently developed using BCIP/NBT substrate (Sigma-Aldrich), and the IFN-γ spots were enumerated with a Bioreader-4000 (BioSys. Karben, Germany). In parallel, unsorted splenic CD8$^+$ T-cells were incubated with anti-CD3ε or BSA coated beads for 6 hrs. The stimulated cells were then stained with the indicated cell surface markers, fixed and permeabilized with BD Cytofix/Cytoperm buffer, and then counter stained with anti-IFN-γ (mAb: XMG1.2).

ADOPTIVE TRANSFERS

Spleen cells prepared from C57Bl/6 mice >40 days following primary infection with LCMV-Armstrong were stained with Alexa 488 conjugated iMap. Spleen cells were subsequently sorted by flow cytomery into Ly-6C$^{\text{iMap(hi)}}$ and Ly-6C$^{\text{iMap(neg)}}$ populations to

>95% purity. To transfer equivalent numbers of $CD8^+$ T-cells, $2x10^6$ Ly-$6C^{iMap(hi)}$ or $1.2x10^7$ Ly-$6C^{iMap(neg)}$ spleen cells were transferred by *i.v.* injection into naïve C57BL/6 mice. The following day, recipient mice were infected *i.p.* with $2x10^5$ PFU LCMV-Armstrong. Four days post infection, recipient mice were euthanized and spleens were harvested.

LCMV IMMUNO-FOCUS ASSAY

Viral titers were determined by an LCMV immuno-focus assay using MC57G cell monolayers. Briefly, spleens were weighed, homogenized using tissue grinders in 2mL of DMEM culture medium and immediately frozen at -80°C. Samples were thawed at 37°C and serial dilutions were prepared in DMEM culture medium. 200μL of each dilution was mixed with $1.4x10^5$ MC57G cells in 200μL of culture medium in 24-well tissue culture plates. Following incubation for 4-6 hrs at 37°C, 100μL of a 1% methylcellulose overlay was added to each well. Two days later, monolayers were washed, fixed and permeabilized with BD Cytofix/Cytoperm buffer. Monolayers were subsequently stained with anti-LCMV (mAb: VL4) hybridoma supernatant, followed by HRP-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratory). Viral foci were detected with O-phenylenediamine substrate and enumerated using a Bioreader-4000 (BioSys).

RESULTS

THE IMAP MONOCLONAL ANTIBODY RECOGNIZES A DISTINCT FORM OF LY-6C.2

We generated an IgM producing hybridoma, iMap, from a BALB/c mouse immunized with C57BL/6 A-LAKs (13). The iMap antibody reacted with C57BL/6, 129/J, AKR/J, C57BL/10 and DBA/2 A-LAKs, but failed to react with those derived from BALB/c, C3H/He, CBA/J and NOD/LtJ (Table 4-1). This particular mouse strain reactivity pattern correlates with the Ly-6.2 haplotype (14). To examine the potential Ly-6 reactivity of iMap, EL4J transfected with Ly-6.2 alleles of Ly-6C or G, and HEK293T transfected with Ly-6.2 alleles of Ly-6A/E, C, or I were stained with iMap. The flow cytometry data showed that iMap only reacted with Ly-6C but not Ly-6A/E, Ly-6G or I (Fig. 4-1*A*). Although these results indicated that iMap recognizes Ly-6C, the iMap reactivity pattern did not correlate exactly with that of AL-21, which is known to react with a non-allelic determinant epitope on Ly-6C (7, 14). For example, both the T-hybridoma RF33.70, and its Ly-6C^{iMap+} RF+ subline obtained by iMap immuno-selection, expressed abundant Ly-6C as detected by AL-21 staining, yet iMap only reacted with RF+, not the bulk RF33.70 population (Fig. 4-1*B*). The differential anti-Ly-6C staining pattern of AL-21 and iMap is not unique to the RF33.70 T-hybridoma, as distinct anti-Ly-6C reactivity is also observed with RMA and MDAY-D2 T-lymphomas (Fig. 4-1*B*). It is highly unlikely that the differential reactivity patterns are due to different affinities of these antibodies, as iMap reactivity with RF33.70 which expressed high levels of Ly-6C recognized by AL-21 was negligible. The AL-21 mAb is not known to react with other Ly-6 molecules and its reactivity can be completely blocked by other anti-Ly-6C mAbs, while iMap reactivity is dependent on Ly-6C.2 expression ((6, 7), Fig. 4-1A). The mature Ly-6C protein is a relatively small molecule consisting of approximately eighty amino acids. Based on their predicted amino acid sequences, there are only two amino acid differences between the two Ly-6C allotypes (15, 16). Yet multiple Ly-6C species with a wide range of *p*Is has been detected by two-dimensional polyacrylamide gel electrophoresis analysis (12). The origin of Ly-6C microheterogeneity is not known, as Ly-6C contains no *N*-linked glycosylation sites; however, the contribution of *O*-linked carbohydrate in this regard has not been ruled out. Regardless, our results indicate that iMap is likely recognizing a different isoform or subset of Ly-6C from that recognized by the AL-21 mAb.

EXPRESSION OF IMAP AND AL-21 REACTIVE LY-6C ON EX VIVO HEMATOPOEITIC CELLS

Since iMap and AL-21 exhibit different Ly-6C reactivity patterns on T-cell lines, we next examined whether differences could be observed on *ex vivo* cells isolated from hematopoietic compartments including BM, thymus, spleen and LN. Gr1$^+$ BM granulocytes are known to express Ly-6C (17). As expected, Gr-1$^+$ BM granulocytes were AL-21 and iMap reactive, by contrast both mAbs failed to react with thymocytes (Fig. 4-2*A*). As for resting peripheral T-cells, iMap appeared to preferentially recognize a subset of splenic and lymph node CD8$^+$ T-cells (Fig. 4-2*B*). In fact, greater than 95% of the Ly-6C^{iMap+} T-cells were CD8$^+$, whereas CD4$^+$ T-cells were almost entirely Ly-6C^{iMap-} (Fig. 4-2*B*). Within the CD8$^+$ T-cell compartment, 30-40% of the T-cells were Ly-6C^{iMap+} (Fig. 4-2*B*). In contrast, Ly-6C^{AL-21} showed a broader distribution, as a distinct and substantial subset of CD4$^+$ in both splenic and lymph node T-cells reacted with this mAb (Fig. 4-2*B*). This observation is in agreement with the previously published data showing that AL-21 recognized a subset of CD4$^+$ T-cells (18-20). In this regard, iMap is similar to another anti-Ly-6C.2 mAb, 143-4-2, which only recognizes peripheral CD8$^+$ Ly-6C$^+$ T-cells (21). As for AL-21, it is similar to 6C3 and 15.1.4.1 pan-Ly-6C mAbs which are capable of reacting with both CD4$^+$ and CD8$^+$ Ly-6C$^+$ T-cells in an allele non-specific manner (18, 22). Whether the reactivity of these mAbs can be classified into these two categories remains to be seen. However, the data suggest that Ly-6C exists in at least two different forms, Ly-6C^{iMap} and Ly-6C^{AL-21}. More importantly, differential Ly-6C^{iMap} and Ly-6C^{AL-21} expression is obvious on mature resting CD4$^+$ and CD8$^+$ T-cells, but not on BM granulocytes and thymocytes.

HIGH EXPRESSION OF LY-6C^{IMAP} IDENTIFIES CD8$^+$ MEMORY T-CELLS

Expression of Ly-6C has been used as a memory marker for CD8$^+$ T-cells and it was assumed that all Ly-6C$^+$ CD8$^+$ T-cells were memory T-cells (9). However, close to fifty percent of resting splenic and LN CD8$^+$ T-cells express Ly-6C whether detected with iMap or AL-21 mAbs (Fig. 4-2*B*). Since memory CD8$^+$ T-cells constitute only a small percentage of total CD8$^+$ T-cells, it is unlikely that half of the resting CD8$^+$ T-cells are memory cells. Given that Ly-6C expression has been reported to be induced on activated T-cells by anti-CD3ε stimulation, it was possible that the Ly-6C$^+$ splenic CD8$^+$ T-cells represent a population of activated cells (23, 24). To clarify this issue, splenic CD8$^+$ T-cells were analyzed for Ly-6C expression with other early activation markers. The Ly-6C^{iMap+} splenic CD8$^+$ T-cells did not co-express CD25, CD40L, CD69 and CD70 (Fig. 4-3*A*). As these markers are transiently expressed on activated T-cells, this suggests that Ly-6C^{iMap+} CD8$^+$ T-cells are not recently activated.

Since the Ly-6C^{iMap+} CD8$^+$ T-cells are not recently activated, we next evaluated whether Ly-6C^{iMap+} cells displayed a memory phenotype. To address this issue, resting splenic T-cells from adult mice were co-stained with anti-Ly-6C^{iMap} and T-cell memory markers. Based upon differential expression of CD44 and CD62L, T-cells can be subdivided into three populations: naïve, T$_{CM}$ and T$_{EM}$ (25, 26). Naïve T-cells are typically CD44^{low}CD62L^{high}, whereas T$_{CM}$ are CD44^{high}CD62L^{high} and T$_{EM}$ are CD62L^{low}CD44^{high}. Resting CD8$^+$ T-cells expressed Ly-6C^{iMap} at varying levels and not all of the Ly-6C^{iMap+} CD8$^+$ T-cells, as detected by iMap, exhibited a memory phenotype (Fig. 4-3*B*). For instance, among Ly-6C^{iMap+} CD8$^+$ T-cells, only the cells that expressed a high level of Ly-6C^{iMap} (Fig. 4-3*B*, gate P2) had a T$_{CM}$ phenotype. As the Ly-6C^{iMap} expression level decreased (Fig. 4-3*B*, gate 3 to gate 8), so did the CD44 expression level and the Ly-6C^{iMap+} CD8$^+$ T-cells began to demonstrate a naïve phenotype. Taken together, these results demonstrate that expression of Ly-6C^{iMap} identifies a population of CD8$^+$ T-cells that are not recently activated and only high expression of Ly-6C^{iMap} correlates with a memory phenotype.

To further confirm that high expression of Ly-6C^{iMap} correlated with T-cells with a memory phenotype, splenic T-cells were cultured in the presence of IL-15, which is known to be required for maintaining memory CD8$^+$ T-cell homeostasis (27). Culture of splenic T-cells in IL-15 maintained a population of cells with high expression of Ly-6C^{iMap}

and a predominantly T_{CM} phenotype (Fig. 4-3*C*, gate P2). By contrast after IL-15 culture, cells gated on lower levels of Ly-6C^{iMap} (Fig. 4-3*C*, gate P3-P7), were of much less proportion compared to Ly-6C$^{iMap(hi)}$ CD8^{+} T-cells than that observed with freshly isolated splenic CD8^{+} T-cells (Fig. 4- 3*B* and *C*). Furthermore, as the Ly-6C^{iMap} expression decreased, CD8^{+} T-cells with a naïve phenotype were more prominent (Fig. 4-3*C*, gate P3-P7) and the majority of CD8^{+}Ly-6C^{iMap-} cells were naïve cells (Fig. 4-3*C*, gate P8). We conclude that high expression of Ly-6C^{iMap} identifies a population of CD8^{+} T-cells that are not recently activated, that express memory cell markers, and are preferentially maintained by IL-15, a cytokine known to sustain memory CD8^{+} T-cells.

LY-6C EXPRESSION KINETICS DURING ACUTE LCMV INFECTION

Our preceding data indicated that Ly-6C^{iMap} expression correlates with CD8^{+} T-cells having a memory phenotype; however, the kinetics of Ly-6C^{iMap} expression during a primary response to acute viral infection has not been addressed. To investigate this issue, C57BL/6 mice were infected with LCMV and the Ly-6C expression was analyzed during the normal course of infection. The CD8^{+} T-cell response to acute viral infection can be divided into three distinct phases: (*i*) the activation and expansion phase (days 1-8 post-infection); (*ii*) the contraction phase in which effector T-cells begin to die (day 8-30); (*iii*) and the memory phase (>30 days) (28). To examine Ly-6C expression on activated T-cells during the three phrases of response to LCMV infection, T-cells from spleens and LNs were isolated at different time points post-infection and stained with iMap. Both CD4^{+} and CD8^{+} T-cells expressed Ly-6C^{iMap} during the early activation and expansion phases of the *in vivo* immune response to LCMV infection (Fig. 4-4*A*). However, compared to CD8^{+} T-cells, CD4^{+} T-cells exhibited a lower percentage of cells with Ly-6C^{iMap} expression. For example, approximately 44% of CD4^{+} T-cells were Ly-6C^{iMap+} at 3 days post-infection, whereas about 90% CD8^{+} T-cells were Ly-6C^{iMap+} (Fig. 4-4*A*). By day 6, the percentage of Ly-6C^{iMap+} on both T-cell subsets increased further, and more than 90% of CD8^{+} T-cells were Ly-6C^{iMap+} (Fig. 4-4*A*). These results showed that the frequency of Ly-6C^{iMap+} T-cells is rapidly enhanced for CD4^{+} and CD8^{+} T-cell subsets upon virus infection. Interestingly, by day 9 and in the early contraction phase, most of the CD4^{+} T-cells had reverted to the Ly-6C^{iMap-} phenotype (Fig. 4-4*B*). By 8 to 10 days post-infection LCMV is normally cleared (28). Thus, CD4^{+} T-cells only expressed Ly-6C^{iMap} during the early phase of the infection and lost Ly-6C^{iMap} expression once the virus was cleared from the system. In sharp contrast, CD8^{+} T-cells could sustain Ly-6C^{iMap} expression at high levels, with greater than 70-80% of CD8^{+} T-cells being Ly-6C^{iMap+} at 9 and 19 days post-infection (Fig. 4-4*A*). By day 32 post-infection and in the memory phase, the percentage of Ly-6C^{iMap+} in both T-cell compartments had returned essentially to the base line level as seen in the uninfected control splenic T-cells. Taken together, these results demonstrated that Ly-6C expression is modulated on T-cells during the course of LCMV infection.

Our kinetic study showed that Ly-6C^{iMap} is expressed at a much higher frequency of CD8^{+} T-cells upon exposure to LCMV. However, it did not determine whether antigen specific responding CD8^{+} T-cells are among the Ly-6C^{iMap+} T-cells. To address this issue, splenic T-cells from LCMV infected mice were analyzed for expression of Ly-6C^{iMap}, CD44 and H-2D^{b} tetramers bound with an immuno-dominant LCMV peptide gp$_{33-41}$. On day 7 post-infection, a small population of H-2D^{b}-gp33 tetramer positive CD8^{+} T-cells could be detected (Fig. 4-5*A*, gate P2). Compared with the total T-cell population, all tetramer positive cells were CD44^{high}Ly-6C$^{iMap(hi)}$ during the acute phase (day 7 post-infection) (Fig. 4-5*A*), demonstrating that Ly-6C^{iMap} is expressed on activated antigen specific T-cells. Similarly, essentially all the H-2D^{b}-gp33 tetramer positive CD8^{+} T-cells detected on day 19 and day 54 post-infection also exhibited the CD44^{hi}Ly-6C$^{iMap(hi)}$ phenotype (Fig. 4-5*B* and *C*). Since the virus is cleared from the animal before day 19, it appears that Ly-6C^{iMap} expression is maintained by the antigen experienced cells. Taken together, these results demonstrated that Ly-6C expression is modulated on T-cells and antigen specific memory CD8^{+} T-cells maintain high expression of Ly-6C^{iMap} well into the memory phase following acute viral infection with LCMV.

LY-6C^{iMAP} EXPRESSION CORRELATES WITH IFN-γ PRODUCTION BY LCMV-SPECIFIC MEMORY CD8^{+} T-CELLS

To further examine the antigen specificity and functionality of Ly-6C^{iMap+} CD8^{+} T-cells, total splenic CD8^{+} T-cells from mice infected with LCMV for >70 days were sorted into four populations based on their Ly-6C^{iMap} expression levels; Ly-6C$^{iMap(neg)}$, Ly-6C$^{iMap(lo)}$, Ly-6C$^{iMap(int)}$ and Ly-6C$^{iMap(hi)}$ (Fig. 4-6*A*). The ability of these sorted Ly-6C^{iMap+} CD8^{+} T-cells to secrete IFN-γ upon antigen stimulation with peptide pulsed target cells was determined by ELISPOT. Compared to naïve CD8^{+} T-cells, memory CD8^{+} T-cells rapidly produce IFN-γ upon antigen stimulation (29, 30). The ELISPOT results showed that LCMV-specific IFN-γ producing cells could only be detected in the Ly-6C$^{iMap(hi)}$ and Ly-6C$^{iMap(int)}$ populations, with the former by far containing the highest number of IFN-γ secreting cells (Fig. 4-6A). In contrast, essentially no LCMV specific IFN-γ producing cells were detected in Ly-6C$^{iMap(lo)}$ and Ly-6C$^{iMap(neg)}$ populations (Fig. 4-6*A*). The IFN-γ production is antigen specific, as these CD8^{+} T-cells failed to secret IFN-γ when stimulated with an irrelevant influenza NP366 peptide (Fig. 4-6*A*). Altogether, these data showed that high expression of Ly-6C^{iMap}, greater than 10,000 relative fluorescence units in our experiments, exhibited by cells in the major peak of Ly-6C^{iMap} staining (Ly-6C$^{iMap(int)}$ and Ly-6C$^{iMap(hi)}$) correlates directly with the cells mediating a functional memory response, rapid IFN-γ production. As the Ly-6C^{iMap} expression level decreased, so did the number of IFN-γ producing cells. In addition to ELISPOT, the intracellular IFN-γ of unsorted CD8^{+} T-cells stimulated with anti-CD3ε coated beads was also determined by flow cytometry. This will ensure that the anti-CD3ε treatment will stimulate the majority of CD8^{+} T-cells in the unsorted population. In agreement with the ELISPOT data with different CD8^{+} T-cell subsets based on Ly-6C^{iMap} expression level, only CD8^{+}Ly-6C$^{iMap(hi)}$ (>10,000 relative fluorescence units in our experiments) produced IFN-γ as detected by the single cell flow cytometry cytokine assay (Fig. 4-6*B*). No IFN-γ production was detected by CD8^{+} T-cells stimulated with BSA-conjugated control beads (Fig. 4-6*B*). These observations taken together with data obtained from resting splenic

T-cells show that the Ly-6C$^{iMap(hi)}$ phenotype can be used to identify functional CD8^{+} memory T-cells.

ADOPTIVE TRANSFER OF LY-6C$^{IMAP(HI)}$ SPLEEN CELLS CONFERS PROTECTION AGAINST LCMV CHALLENGE

Thus far, Ly-6C^{iMap} expression has been analyzed using enriched splenic and LN T-cells. Using Alexa 488-conjugated iMap, flow cytometric analysis was performed on whole spleen cell preparations to determine its pattern of expression. High expression of Ly-6C^{iMap} was found predominantly on CD8^{+} T-cells. Minimal or no iMap staining was found on CD4^{+} T-cells and B-cells, whereas NK-cells expressed intermediate levels of Ly-6C^{iMap} (Fig. 4-7*A*). Since acute LCMV clearance is mediated primarily by CD8^{+} T-cells, we sought to determine if Ly-6C$^{iMap(hi)}$ spleen cells could confer protective immunity against LCMV challenge following adoptive transfer (28). To this end, spleen cells from LCMV immune mice (>90 days post infection) were stained with iMap and sorted into Ly-6C$^{iMap(hi)}$ and Ly-6C$^{iMap(neg)}$ populations. The Ly-6C$^{iMap(hi)}$ population consisted of ~60% CD8^{+} T-cells which had a corresponding memory phenotype. In comparison, the Ly-6C$^{iMap(neg)}$ population was comprised of ~10% CD8^{+} T-cells, the majority of which were phenotypically naïve (Fig. 4-7*B*). To ensure equivalent numbers of CD8^{+} T-cells were transferred, $2x10^{6}$ Ly-6C$^{iMap(hi)}$ spleen cells and $1.2x10^{7}$ Ly-6C$^{iMap(neg)}$ spleen cells were injected *i.v.* into naïve C57BL/6 mice. Control mice received PBS injections. The following day, the recipient mice received *i.p.* injections of $2x10^{5}$ PFU LCMV-Armstrong and viral titers in the spleen were determined four days post-infection. Both the control and the mice that received Ly-6C$^{iMap(neg)}$ spleen cells had high titers of LCMV in their spleens. In contrast, mice that received Ly-6C$^{iMap(hi)}$ spleen cells had significantly reduced splenic viral titers that were almost below the limit of detection (Fig. 4-7*C*). Thus, Ly-6C$^{iMap(hi)}$ spleen cells from LCMV immune mice can confer protection against LCMV challenge following adoptive transfer into naïve hosts. Other cell surface antigens such as CD44, CD122 and CD45 are broadly expressed by both CD4^{+} and CD8^{+} T-cells in addition to various other lymphocytes. Therefore, iMap is distinct in its ability to enrich functional antigen-specific memory CD8$^+$ T-cells from total spleen and LN preparations.

DISCUSSION

Due to their unique and varied distribution patterns, Ly-6C molecules have been used as differentiation markers for hematopoietic cells. Relevant to the present study, Ly-6C expression has been associated with CD8$^+$ T-cell activation and identified as a CD8$^+$ T-cell memory marker (9). However, Ly-6C recognizing antibodies do not uniformly cross-react and little is understood about the relationship of Ly-6C expression and effector function of memory CD8$^+$ T-cells *in vitro* and *in vivo*. We have clarified some of these issues, particularly in the context of a novel mAb we generated that recognizes a Ly-6C.2 epitope that when highly expressed, directly identifies functional memory CD8$^+$ memory cells in resting secondary lymphoid organs.

In the present study, we found that iMap exhibited partially overlapping, yet distinct anti-Ly-6C reactive patterns compared to the non-allelic Ly-6C epitope recognized by AL-21. This differential reactivity of anti-Ly-6C mAbs suggests the existence of different forms of Ly-6C. For instance, Ly-6C$^{AL\text{-}21}$ present on the RF33.70, RMA and MDAY-2 cell lines was not detected by iMap. Furthermore, both iMap and AL-21 detected high levels Ly-6C on Gr-1$^+$ BM granulocytes, whereas Takikawa *et al.* isolated an anti-Ly-6C mAb that does not to react with Gr-1$^+$ BM granulocytes (12). They also showed that Ly-6C isolated from macrophages exhibited a wide range of *pI*s, suggesting Ly-6C possesses microheterogeneity (12). Together, these data confirm the existence of different forms of Ly-6C and each cell type might express a unique set of Ly-6C isoforms.

From our analysis of Ly-6C expression during LCMV infection, there are clear differences between the expression of Ly-6C^{iMap} and Ly-6C$^{AL\text{-}21}$ on T-cell subsets. For instance, upon LCMV infection a large percentage of Ly-6C^{iMap+} CD4$^+$ T-cells could be detected. However, in comparison to Ly-6C^{AL-21}, CD4^{+} T-cells failed to sustain Ly-6C^{iMap} expression. In the memory phase of the primary immune response, little or no Ly-6C^{iMap} could be detected on CD4^{+} T-cells. On the other hand, half of the CD4^{+} T-cells were positive for Ly-6C^{AL-21} prior to viral infection and following clearance of the virus. This observation is slightly different from the *in vivo* system in which pigeon cytochrome C was used as an antigen. In this case, it was reported that Ly-6C^{AL-21} expression is lost early on after *in vivo* pigeon cytochrome C activation (20). The reason for this discrepancy is not known, but it could be due to the nature of the antigen used in these studies since LCMV infection would induce significantly more inflammation as compared to a peptide antigen and adjuvant. Nevertheless, in the LCMV infection model, different forms of Ly-6C on the CD4^{+} T-cell compartment are regulated differently; Ly-6C^{iMap} being inducible and Ly-6C^{AL-21} being constitutively expressed. This observation raises an interesting question regarding the function of Ly-6C^{iMap+} CD4^{+} T-cells. Since Ly-6C^{iMap+} CD4^{+} T-cells could only be sustained for a short period of time during the acute phase of the viral infection, it is difficult to envision Ly-6C^{iMap+} CD4^{+} T-cells are involved in the development of antibody producing plasma cells as those demonstrated for Ly-6C^{AL-21+} CD4^{+} T-cells (20). Recently, high expression of Ly-6C on CD4^{+} T-cells have been used to identify T-helper cells able to support early plasma cell development *in vivo* (20). Therefore, it would be interesting to examine whether during the course of infection, do Ly-6C^{iMap-} and Ly-6C^{iMap+} CD4^{+} T-cells have differential functional properties. For instance, it is not unreasonable to assume that Ly-6C^{iMap+} and Ly-6C^{iMap-} T-cells represent two developmental pathways that lead to distinct cytokine production profiles. Regardless, our data showed that the regulation of Ly-6C is more complex than previously thought.

In addition to demonstrating that CD4^{+} T-cells could express Ly-6C^{iMap}, the kinetic experiments importantly showed that Ly-6C$^{iMap(hi)}$ expression on antigen specific CD8^{+} T-cells was sustained well into the memory phase of the immune response. The presence of LCMV specific Ly-6C$^{iMap(hi)}$ memory CD8^{+} T-cells was confirmed by their ability to secrete rapidly IFN-γ upon antigen stimulation. Essentially, these data agree with our other observation that only resting Ly-6C$^{iMap(hi)}$ CD8^{+} T-cells and IL-15 maintained T-cells exhibited memory phenotypes and function. The data also showed that caution should be exercised when using Ly-6C as a T-cell memory marker, as not all Ly-6C^{+} CD8^{+} T-cells are functional memory T-cells. This was demonstrated by the fact that as Ly-6C^{iMap} expression levels decreased on CD8^{+} T-cells, the ability to secrete IFN-γ also decreased. Although memory T-cells are not homogeneous populations and can be divided into T$_{CM}$ and T$_{EM}$ populations, phenotypic analysis showed that both types of memory T-cells were Ly-6C$^{iMap(hi)}$. However, the conditions required to maintain Ly-6C$^{iMap(hi)}$ expression on these memory cells are currently unknown. Given the evidence for different forms of Ly-6C, it would be interesting to examine whether certain forms of Ly-6C are preferentially expressed by, or distinguish between, T$_{CM}$ and T$_{EM}$ T-cells. Unlike CD8^{+} T-cells, little or no Ly-6C^{iMap+} CD4^{+} T-cells could be detected in the memory phase. This strongly implies that Ly-6C carries out different functions in CD4^{+} and CD8^{+} T-cells. Since Ly-6C is known to be involved in endothelial adhesion and homing of CD8^{+} T-cells, it is not unreasonable to speculate that Ly-6C^{iMap} might possess similar function and facilitate memory T-cells to home to certain lymphoid compartments (7). Recently, it was shown that the gut microenvironment supports the differentiation of a unique Ly-6C^{low} CD8^{+} T-cell population (11). It is noteworthy that Ly-6C might not be downregulated on these gut CD8^{+} T-cells, but rather they express a unique form of Ly-6C. It would be interesting to examine whether Ly-6C^{iMap} and other Ly-6Cs are maintained or down-regulated on these gut memory T-cells. It is interesting to note the correlation of Ly-6C$^{iMap(hi)}$ and memory T-cells is similar to those reported for Ly-6C expression on macrophage progenitors. For instance, among the splenic CD4^{-}CD8^{-}sIg^{-} populations which showed a heterogeneous Ly-6C expression pattern, only the small Ly-6C^{high} population contained the macrophage progenitors (6). Therefore, a more detailed analysis should be carried out on the expression of serologically distinct forms of Ly-6C on different hematopoeitic cell types.

The identification of phenotypic markers to identify functional memory CD8$^+$ T-cells has consistently challenged researchers. Therefore, our finding that iMap can be used as a single phenotypic marker to enrich memory CD8$^+$ T-cells is a significant discovery. Other commonly used memory markers such as CD44, CD62L, CD45 and CD127 can be used to identify memory CD8$^+$ T-cells, but they are also expressed by large percentages of other lymphocyte populations such as CD4$^+$ T-cells and B-cells. Furthermore, their expression can by dynamically regulated during various stages of differentiation, much like Ly-6C. However, Ly-6C^{iMap} is unique since it is predominantly found on CD8$^+$ T-cells and high expression correlates with a functional memory phenotype. This was clearly demonstrated by our adoptive transfer experiments using Ly-6C$^{iMap(hi)}$ as the only marker to identify LCMV-specific memory CD8$^+$ T-cells from the spleens of immunized mice. The Ly-6C$^{iMap(hi)}$ spleen cells contained mostly CD8$^+$ T-cells bearing a memory phenotype and could protect against LCMV challenge upon adoptive transfer into naïve hosts. Taken together, iMap may prove to be a very useful reagent to enrich and isolate memory CD8$^+$ T-cells from secondary lymphoid organs.

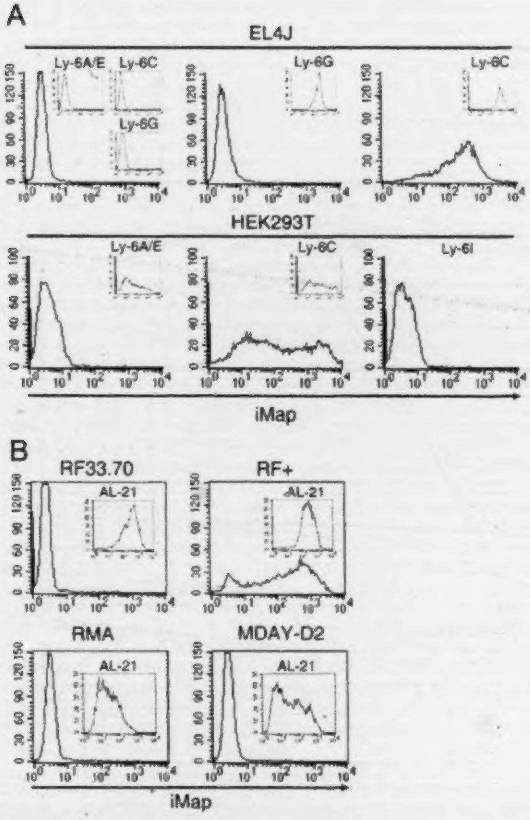

Figure 4-1. iMap recognizes the Ly-6C.2 protein. A) Untransfected EL4J (top left panel) or EL4J and HEK293T transfected with Ly-6A/E, C and G or I were stained with iMap. The inset FACS plots showed the expression level of the indicated Ly-6 by each transfectant. Ly-6 A/E, C, and G were detected by mAb D7, AL-21, and RB6-8C5, respectively. No Ly-6I reactive antibody is commercially available. B) Differential Ly-6C expression as detected by iMap and AL-21 mAbs. Expression of Ly-6C$^{\text{iMap}}$ on the parental RF33.70 and its Ly-6C$^{\text{iMap+}}$ subline RF+, RMA and MDAY-D2 were detected by iMap. The inset FACS plots showed the Ly-6C expression on the indicated cell lines as detected by AL-21.

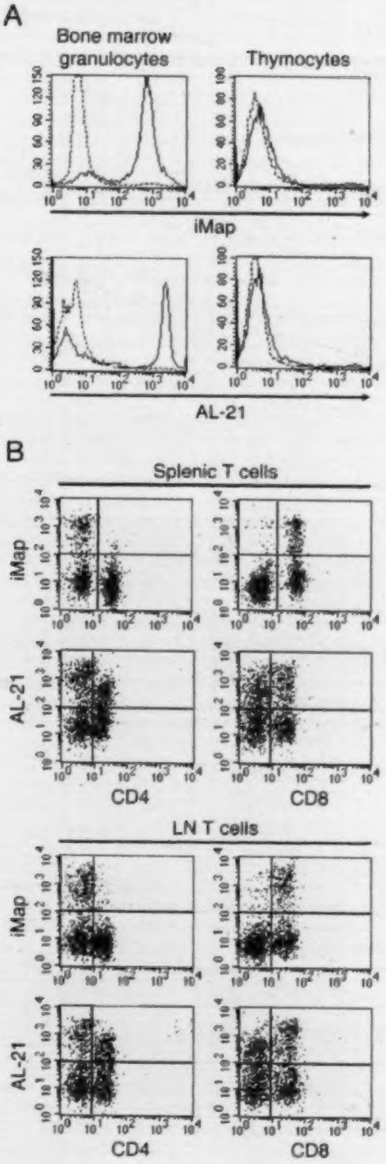

Figure 4-2. Expression of Ly-6C^{iMap} and Ly-6C^{AL-21} on C57BL/6 resting *ex vivo* hematopoeitic cells. A) Gr-1^{+} BM granulocytes and thymocytes were incubated with iMap or AL-21 followed by PE-conjugated anti-mouse/rat IgM. Dashed and solid lines represent isotype controls (B27M2 or DX5) and anti-Ly-6C (iMap or AL-21) staining, respectively. B) Distribution of Ly-6C^{iMap} and Ly-6C^{AL-21} on resting splenic and lymph node T-cells. T-cells isolated from spleens and LNs were stained with anti-Ly-6C (iMap or AL-21) and anti-CD4 or anti-CD8α.

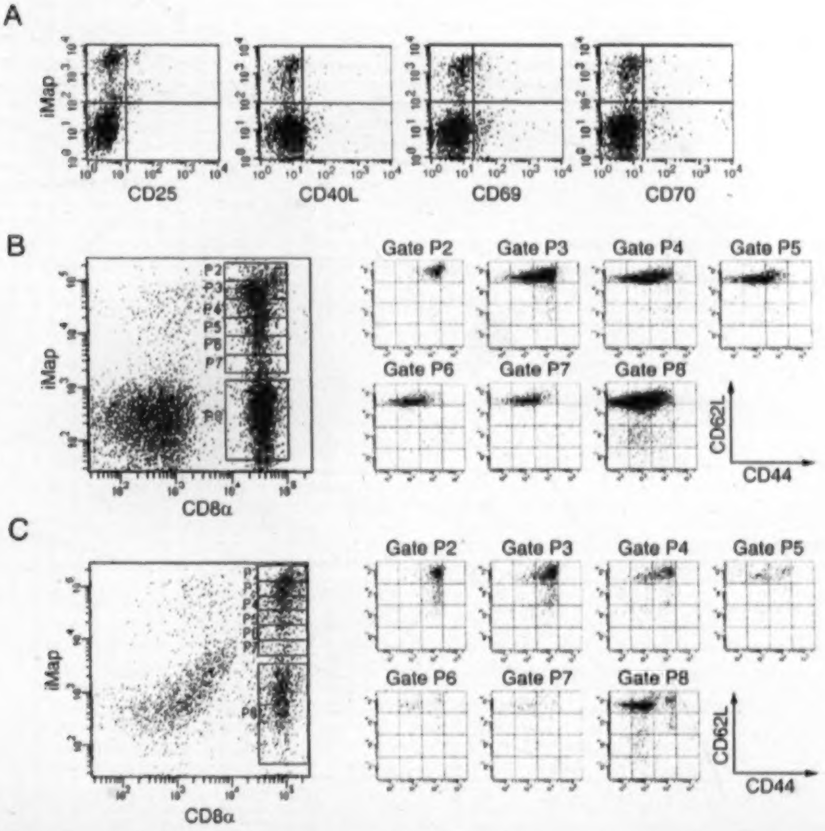

Figure 4-3. Phenotypic analysis of Ly-6C^{iMap+} T-cells and the correlation of Ly-6C^{iMap} expression with memory T-cell phenotypes. A) Resting splenic T-cells were co-stained with anti-Ly-6C^{iMap} and anti-CD25, CD49L, CD69 or CD70. B) Multi-paramter analysis of splenic T-cells for the expression of Ly-6C^{iMap} and T-cell memory specific markers. (Left panel) Ly-6C^{iMap} expression level specific gates were set on splenic CD8^{+} T-cells; gate P2 to P8. (Right panels) Each cell population within gates P2 to P8 was analyzed for the expression of the T-cell phenotypic markers CD44 and CD62L. (C) *In vitro* IL-15 differentiated memory T-cells were analyzed as above.

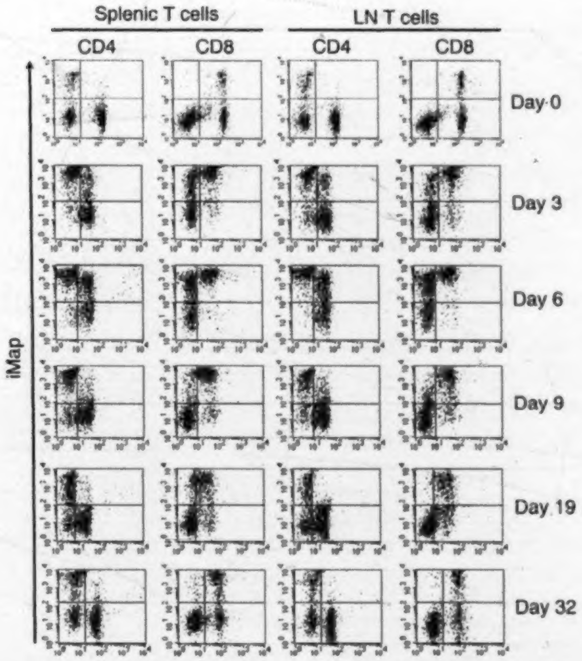

Figure 4-4. ***In vivo* activated CD4$^+$ and CD8$^+$ T-cells express high levels of Ly-6C.** Kinetics of Ly-6C expression on CD4$^+$ and CD8$^+$ T-cells isolated during LCMV infection. Ly-6C expression was analyzed on CD4$^+$ and CD8$^+$ T-cells isolated from spleens and LNs of C57BL/6 mice infected with LCMV for 0, 3, 9, 19, and 32 days using the iMap mAb. In all instances, Ly-6C^{iMap} expression is indicated on the *y*-axis, whereas the *x*-axis indicates either CD4 or CD8 staining.

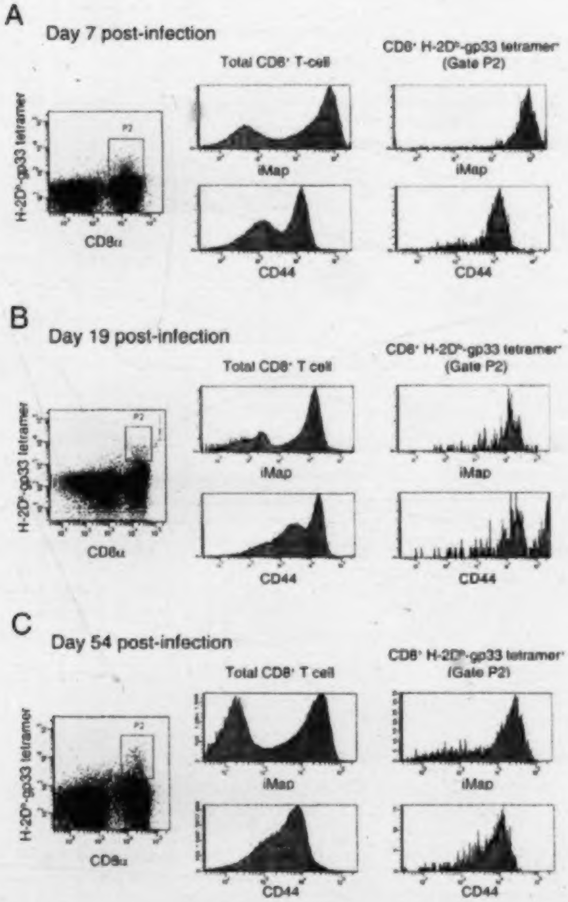

Figure 4-5. Ly-6C^{iMap+} T-cells exhibited antigen specificity against LCMV. Correlation of H-2D^{b}-gp33 tetramer specificity with CD44 and Ly-6C^{iMap} expression on splenic T-cells isolated from mice infected with LCMV on days 7, 19 and 54 post-infection. A) On day 7, a small population of CD8^{+} T-cells showed positive staining with H-2D^{b}-gp33 tetramers (boxed population, gate P2) (Left). The histogram plots indicate the Ly-6C^{iMap} and CD44 expression levels on the total splenic T-cell populations (Right). The gated H-2D^{b}-gp33 tetramer positive CD8^{+} T-cells exhibited a Ly-6C$^{iMap(hi)}$CD44^{high} phenotype. B) Day 19 post-infection. C) Day 54 post-infection.

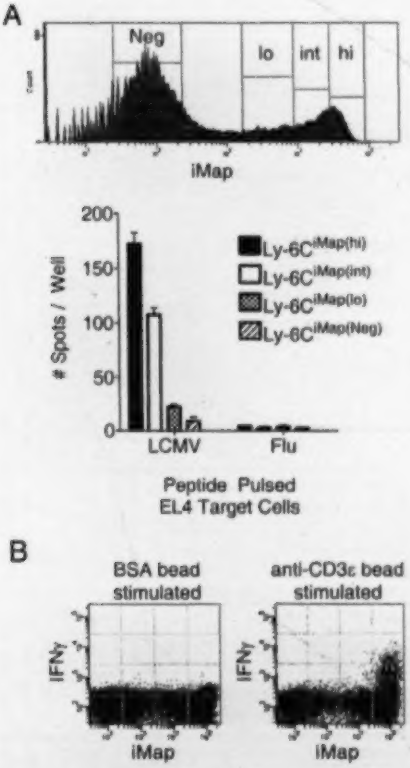

Figure 4-6. IFN-γ producing memory CD8$^+$ T-cells express a high level of Ly-6C^{iMap}. A) Splenic T-cells from mice infected with LCMV for >70 days were sorted into Ly-6Ci$^{Map(neg)}$, Ly-6C$^{iMap(lo)}$, Ly-6C$^{iMap(int)}$ and Ly-6C$^{iMap(hi)}$ populations as indicated by the histogram plot. The sorted cells were stimulated with LCMV peptide pulsed EL4 cells and an IFN-γ ELISPOT assay was performed as described in *Materials and Methods*. B) Unsorted total splenic T-cells were stimulated with BSA (control) or anti-CD3ε coated beads for 5 hrs. The presence of intracellular IFN-γ and their correlation with Ly-6C^{iMap} expression levels was detected by flow cytometry.

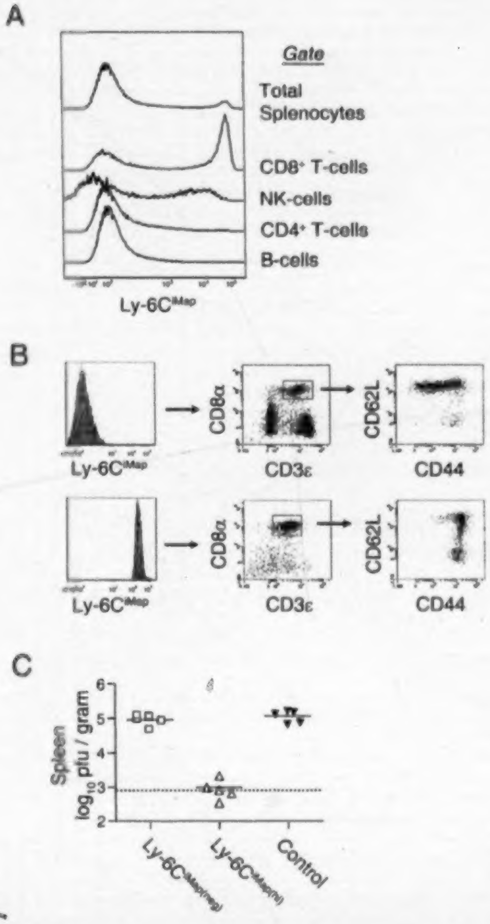

Figure 4-7. Ly-6C$^{iMap(hi)}$ can be used as a single marker to identify functional memory CD8$^+$ T-cells. Whole spleen cells from LCMV immune mice (>90 days post infection) were stained with iMap and various other cell specific markers. A) The majority of Ly-6C$^{iMap(hi)}$ spleen cells were CD8$^+$ T-cells. B) CD8$^+$ T-cells within the FACS sorted Ly-6C$^{iMap(hi)}$ and Ly-6C$^{iMap(neg)}$ populations was analyzed for CD44 and CD62L expression. (C) Ly-6C$^{iMap(hi)}$ or Ly-6C$^{iMap(neg)}$ spleen cells or PBS (control), was transferred by *i.v.* injection into naïve C57Bl/6 mice. The following day, recipient mice were infected *i.p.* with 2x10^5 PFU LCMV-Armstrong. Four days post-infection, viral titers in the spleen were determined by an immuno-focus assay. Dashed line indicates the limit of detection.

Table 4-1. The expression profiles of iMap on A-LAKs and bone marrow granulocytes derived from different mouse strains correlates with Ly-6 alloantigen haplotype expression patterns

Mouse strain	iMap reactivity on A-LAKs[a,b]	Ly-6 haplotype
C57BL/6	++++	Ly-6.2
C57BL/10	++++	Ly-6.2
DBA/2	++++	Ly-6.2
129/J	++++	Ly-6.2
AKR/J	++++	Ly-6.2
BALB/c	-	Ly-6.1
C3H/He	-	Ly-6.1
CBA/J	-	Ly-6.1
NOD/LtJ	-	Ly-6.1

[a] The anti-HLA-B27, B27M2, was used as an isotype control. In all instances, the MFI of the isotype control samples was between 3 to 6 relative log fluorescence units.

[b] When positive staining of A-LAKs was observed, two distinct populations, Ly-6C^{iMap+} and Ly-6C^{iMap-} were detected.

" - "sign indicates no iMap staining was detected.

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CHAPTER 5

GENERAL DISCUSSION

In the last 30 years, there has been remarkable progress in understanding the parameters that govern the dynamics of the CD8$^+$ T-cell response (1, 2). It is becoming increasing clear that cytokines are involved in virtually every aspect of the immune response. Therefore, acquiring a greater understanding of their role in shaping the immune response to various pathogens and malignancies will be essential for the development of effective cellular vaccines and immunotherapeutics (3).

Interleukin-15 is a pleiotropic cytokine that plays an important role in both the innate and adaptive immune system (4-7). Although IL-15 is expressed by a broad spectrum of cell types and tissues, under normal physiological conditions, soluble IL-15 is virtually undetectable in cell culture supernatants or in the circulation (8-20). This can be explained by a complex system of regulatory controls that govern IL-15 transcription, translation and protein trafficking (4-7, 18-20). Expression of the high-affinity IL-15Rα chain is equally complex and shows significant overlap with IL-15 (21, 22). Accordingly, the co-expression of both IL-15 and IL-15Rα is required for the stabilization and cell surface expression of the IL-15Rα:IL-15 complex, which can be presented in *trans* to apposing cells (23-27). In addition, following cell-surface expression, the IL-15Rα:IL-15 complex can be taken up into endocytic vesicles where, rather than being targeted for degradation, it is recycled back to the cell-surface (27). This represents a unique feature of IL-15, allowing its long-term persistence at the cell surface. With its broad cellular and tissue distribution, it is not surprising that IL-15 is involved in a myriad of immune and non-immune related functions (4-7).

Cell-sized microspheres have been used successfully to identify the contribution of individual ligands to complex cellular processes (28-33). Thus, using a microsphere based approach, the specific role of IL-15 transpresentation in various immune processes was examined. In chapter 2, it was demonstrated that IL-15 transpresentation has a pronounced effect on the selective binding of $CD8^+$ T-cells to microspheres bearing immobilized IL-15Rα:IL-15 complexes. The signals provided by transpresented IL-15 mediated adhesion were found to be sufficient for the long-term *in vitro* maintenance of antigen-specific memory $CD8^+$ T-cells. In chapter 3, the role of IL-15 transpresentation in the augmentation of memory $CD8^+$ T-cell recall responses was examined. It was found that IL-15 transpresentation was required for the optimal recall responses of T_{CM} $CD8^+$ T-cells, whereas T_{EM} $CD8^+$ T-cell responses were largely independent of transpresented IL-15 stimulation. In chapter 4, a novel anti-Ly-6C mAb that identifies memory $CD8^+$ T-cells in secondary lymphoid organs was characterized. How these findings relate to one another and to the current literature will be addressed in the following discussion.

DIVERSE ROLE OF IL-15 TRANSPRESENTATION IN IMMUNE FUNCTION

One of the many functions of IL-15 is to maintain the numbers of memory $CD8^+$ T-cells through the induction of homeostatic proliferation (34, 35). This concept has been strongly supported by the finding that both IL-15 and IL-15Rα deficient mice lack peripheral memory $CD8^+$ T-cells (36, 37). From our studies, it is clear that IL-15 transpresentation mediates the preferential binding and adhesion of lymphocytes (Fig. 2-4). Functionally, this interaction was found to be sufficient for the long-term maintenance of memory $CD8^+$ T-cells *in vitro* (Fig. 2-7). Therefore, due to the widespread cellular distribution of IL-15 and IL-15Rα, any cell capable of transpresenting IL-15, regardless of its lineage may be directly involved in the adhesion and maintenance of lymphocytes.

ROLE OF IL-15 TRANSPRESENTATION IN THE LUNG

Experiments conducted using BM-chimeric mice have demonstrated that the cellular origin of IL-15Rα and IL-15 expression required for memory CD8$^+$ T-cell homeostatic proliferation can vary depending on the tissue location (25). In the spleen, BM-derived cells are required; whereas, in the lung both BM-derived and parenchymal cells contribute to memory CD8$^+$ T-cell maintenance. This suggests that memory CD8$^+$ T-cells can respond to cells of hematopoietic and non-hematopoietic origin that express the IL-15Rα:IL-15 complex. Unlike macrophages and DCs that upregulate IL-15 and IL-15Rα expression upon activation, non-hematopoietic cells in the lung appear to constitutively express IL-15Rα (8, 11, 25, 38-41). It is proposed that upon binding of excess IL-15 from the circulation, non-hematopoietic cells in the lung function as an intracellular reservoir of IL-15 through the endocytic recycling of the IL-15Rα:IL-15 complex (25). Following pathogen clearance, IL-15 retention in the lung can maintain the survival of tissue resident antigen-specific CD8$^+$ T-cells. Since IL-15 can also limit CD8$^+$ T-cell contraction, the retention of IL-15 may also function to maintain increased numbers of antigen specific CD8$^+$ T-cells in the lung (42). This could eventually lead to increased numbers of memory CD8$^+$ T-cells, which in the lung environment predominantly consists of a T_{EM} CD8$^+$ T-cell population (43-46). The maintenance of the lung resident T_{EM} CD8$^+$ T-cells by IL-15 is somewhat contradictory to our results showing that T_{EM} CD8$^+$ T-cells from the spleen are not very responsive to transpresented IL-15 (Fig. 3-5). It would therefore be interesting to examine whether or not T_{EM} CD8$^+$ T-cells in peripheral tissues have differential responsiveness to IL-15 compared to splenic T_{EM} CD8$^+$ T-cells. It has been reported that upon migration into peripheral sites such as the lung, T_{CM} CD8$^+$ T-cells from the spleen acquire a T_{EM} like phenotype (47). The mechanism of this phenotypic alteration is also unknown, but it may account for the ability of lung resident memory CD8$^+$ T-cells with a T_{EM} phenotype to respond to transpresented IL-15. Taken together, it appears that IL-15 transpresentation by both hematopoietic and non-hematopoietic cells is important in maintaining effective long-term survival and homeostatic proliferation of memory CD8$^+$ T-cells in the lung.

ROLE OF IL-15 TRANSPRESENTATION IN LYMPHOCYTE MIGRATION

Cytokines such as IL-2 and IL-12 have been shown to increase adhesion and transendothelial migration of NK-cells and CD8$^+$ T-cells, primary through integrin activation (48-52). As demonstrated in chapter 2, IL-15 transpresentation can mediate the direct adhesion of specific lymphocyte populations, in the complete absence of any additional integrin ligands. Interestingly, inflammatory cytokines such as TNF-α and IFN-γ can induce vascular endothelial cells to express cell-surface IL-15 (51). This results in the enhanced LFA-1 mediated transendothelial migration of NK-cells and T-cells (51, 53). In addition, endothelial cells themselves can respond to IL-15 by expressing hyaluronan, which promotes activated and memory T-cell extravasation via interaction with its ligand CD44 (54). The foregoing results suggest that IL-15 expressed on inflamed vascular endothelial cells can mediate the direct binding of NK-cells and CD8$^+$ T-cells, thereby promoting their extravasation into inflamed tissues. This is very relevant to our findings since we observed that IL-15 transpresentation mediated adhesion is remarkably similar to tethered adhesion mediated by chemokines ((55, 56), Fig. 2-6). However, in contrast to CCL19 and CCL21 that aid the entry of naïve and memory CD8$^+$ T-cells into LNs via high endothelial venules, IL-15 transpresentation may aid in recruiting activated and memory CD8$^+$ T-cells to localized sites of inflammation through the vascular endothelium. Unfortunately, there are limited data available on the recruitment of CD8$^+$ T-cells and NK-cells to sites of localized inflammation in either IL-15 or IL-15Rα deficient mice. Most studies examining tissue localization of activated CD8$^+$ T-cells in IL-15 or IL-15Rα deficient mice have been performed with systemic infections, which do not induce localized tissue inflammation (57, 58). However, the direct injection of IL-15 into the foot pad of mice results in a localized tissue infiltrate that consists of predominantly T-cells (59). The dysregulated expression of IL-15 is now considered as one of the key instigators of rheumatoid arthritis pathogenesis which is an autoimmune disorder characterized by synovial inflammation (60-67).

TRANSPRESENTATION OF IL-15 IN SECONDARY LYMPHOID ORGANS

In chapter 2, we demonstrated that microspheres bearing immobilized IL-15Rα:IL-15 complexes can mediate the rapid binding of CD8$^+$ T-cells and possibly NK-cells (Fig. 2-4). In contrast to CD8$^+$ T-cells that require activation prior to the acquisition of effector function, NK-cells were initially thought to exist in a constitutively activated state (68-70). However, recent evidence suggests that NK-cells are similar to CD8$^+$ T-cells in that they require activation by DCs in secondary lymphoid organs (71). *In vivo* ablation of CD11c$^+$ DCs results in significantly diminished NK-cell responses against viral and bacterial pathogens. Upon further investigation, It was found that transpresentation of IL-15 by DCs was necessary and sufficient for the priming of NK-cells in a cell contact dependent manner. Given that activated NK-cells can be detected in the periphery as early as 8 hrs following infection, it suggests that NK-cells must interact with activated DCs very early in the immune response. Based on our findings, it could be speculated that elevated CD122 expression could provide NK-cells a selective advantage in binding to IL-15 transpresented by activated DCs (Fig. 2-4*D*). In support of this, CD122 blockade prevents the activation of NK-cells by DCs (72).

Similar to NK-cells, CD8$^+$ T-cells require direct cell contact with DCs for their activation (73). Due to the low frequency of antigen specific CD8$^+$ T-cells, successful TCR engagement with cognate pMHC is a very rare event (74). Therefore, CD8$^+$ T-cells are more likely to receive pMHC independent stimulation via transpresented IL-15 following interaction with an activated DC. Naive CD8$^+$ T-cells appear to be largely unresponsive to transpresented IL-15 signals alone, whereas memory CD8$^+$ T-cells are highly responsive to transpresented IL-15. This IL-15 dependent interaction could result in several possible outcomes for a memory CD8$^+$ T-cell. Obviously based on its role in homeostatic proliferation, transpresented IL-15 could provide the minimal signals necessary to maintain the survival of memory CD8$^+$ T-cells (34, 35, 75). Secondly, IL-15 has been found to play a role in the avidity maturation of memory CD8$^+$ T-cells by upregulating the expression of the CD8$\alpha\beta$ heterodimer (76). Since CD8$\alpha\beta$ is a co-receptor for the TCR, elevated levels of CD8$\alpha\beta$ enhances the functional avidity of the memory CD8$^+$ T-cell (77). High avidity memory CD8$^+$ T-cells were shown to have elevated expression of IL-15Rα that provided a competitive advantage for limited amounts of endogenous IL-15. This can lead to the preferential survival of high avidity memory CD8$^+$ T-cells and the increased avidity of the memory CD8$^+$ T-cell population over time (76). Another functional outcome could be the induction of chemokinesis by IL-15 (78). Enhanced random motility following IL-15 stimulation could increase the probability of a CD8$^+$ T-cell finding a DC expressing its cognate antigen pMHC complex (51, 53, 78). Also, as transpresented IL-15 can induce grB expression, it may serve to maintain a heightened state of activation (Fig. 3-4*B*). Finally, if a memory CD8$^+$ T-cell – DC interaction leads to a productive TCR engagement, the transpresented IL-15 could provide costimulatory signals necessary for the full activation of the responding CD8$^+$ T-cell as demonstrated in chapter 3. Interestingly, IL-15Rα has been found to co-localize with MHC class I in lipid rafts (79). This finding was demonstrated in cell lines; however, if this also occurs in DCs, this has direct implications to our model. If transpresented IL-15 and MHC class I were found in close proximity on the cell surface of a DC, it would provide a rapid means to provide IL-15 and TCR signals simultaneously to a responding CD8$^+$ T-cell.

IL-15 TRANSPRESENTATION AND MEMORY CD8$^+$ T-CELL LINEAGE DEVELOPMENT

Regarding the minimal requirements for memory CD8$^+$ T-cell homeostasis, our results from chapter 2 clearly demonstrated that transpresented IL-15 in the complete absence of any additional non-T-cell mediated stimuli could maintain the long-term survival and antigen specificity of memory CD8$^+$ T-cells (Fig. 2-7). It was also evident from our analysis that although the TCR specificity did not appear to be altered in the final 30 days of culture, there appeared to be a shift in the T_{CM} to T_{EM} ratio in the population (Fig. 2-7*B*). In chapter 3, T_{CM} $CD8^+$ T-cells were found to be more responsive to transpresented IL-15 alone and undergo proliferation following IL-15 exposure (Fig. 3-5*D*). This was in contrast to the T_{EM} $CD8^+$ T-cell population that did not seem to respond to any significant extent to transpresented IL-15 alone (Fig. 3-5*C*). Taken together, these results suggest that the increase of T_{CM} $CD8^+$ T-cells observed in the later stages of *in vitro* IL-15 culture could have been the result of T_{CM} $CD8^+$ T-cell expansion and the lack of proliferation by the T_{EM} $CD8^+$ T-cells. However, the death rate of the proliferating cells was not examined in our study. Unfortunately, very little has been reported in the literature regarding the rate of cell death among T_{CM} and T_{EM} $CD8^+$ T-cell populations. Nevertheless, our findings fit with a recent model of memory $CD8^+$ T-cell development, suggesting that terminally differentiated T_{EM} are destined to eventually die rather than undergo a conversion into T_{CM} $CD8^+$ T-cells (80). Recently KLRG-1 and IL-7Rα have become useful markers to distinguish between SLECs and MPECs (81-83). It would therefore be interesting to reexamine the expression patterns of these two markers on the transpresented IL-15 maintained $CD8^+$ T-cells during the course of culture.

ROLE OF IL-15 TRANSPRESENTATION FOR $CD4^+$ T-CELLS AND B-CELLS

Although our studies clearly demonstrated that $CD8^+$ T-cells preferentially bound to microspheres bearing transpresented IL-15, a sizeable proportion of cells bound to IL-15 transpresenting microspheres were either $CD4^+$ T-cells or B-cells (Fig. 2-4). For the most part, it is believed that IL-15 does not have significant effects on $CD4^+$ T-cells and B-cells, since the development and phenotype of both lymphocyte populations appears normal in mice lacking either IL-15 or IL-15Rα (36, 37). However, recent data suggests that IL-15 does play a role in the homeostatic proliferation of $CD4^+$ T-cells (84). In a lymphopenic environment where IL-7 availability is not limited, $CD4^+$ T-cell rely heavily on IL-7 for their survival. However, under normal conditions where endogenous levels of IL-7 are low, $CD4^+$ T-cells have been found to be responsive to endogenous IL-15 (84). The selective removal of CD8$^+$ T-cells and NK-cells increases the responsiveness of CD4$^+$ T-cells to IL-15, which suggests that under normal conditions they are outcompeted for IL-15 by CD8$^+$ T-cells and NK-cells. This is in line with our findings that CD8$^+$ T-cells bind to microspheres transpresenting IL-15 more effectively than CD4$^+$ T-cells and B-cells (Fig. 2-4). Regarding B-cells, in a resting state they appear to be generally unaffected by IL-15. However, in combination with CD40 ligand engagement or surface IgM crosslinking, IL-15 can induce B-cell proliferation and antibody synthesis *in vitro* (85). Therefore, it remains entirely possible that IL-15 transpresentation affects both CD4$^+$ T-cell and B-cells and the functional outcome of these interactions should be studied in more detail.

Taken together, our findings have highlighted a previously unknown functional property of transpresented IL-15 in the adhesion and binding of lymphocytes expressing components of the IL-15 receptor complex. Depending on the cellular origin, tissue environment and the responding lymphocyte population, signals provided by transpresented IL-15 can have diverse functional outcomes. These can range from the homeostasis of memory CD8$^+$ T-cells, activation of NK-cells and CD8$^+$ T-cells as well as regulating lymphocyte migration to inflammatory tissues. In addition, IL-15 has bioregulatory functions beyond those related to lymphocytes such as inhibiting apoptosis of keratinocytes, promoting formation of osteoclast-like cells in BM, and anabolic effects on myocytes (4). Therefore, it is clear that there is significant more to be done to elucidate the complex role of this pleiotropic cytokine on both immune and non-immune cell types.

AUGMENTATION OF CD8$^+$ T-CELL ACTIVATION BY TRANSPRESENTED IL-15

In addition to their defined role in development and homeostasis, cytokines also play an important role in initiating and supporting CD8$^+$ T-cell responses. For example, IL-2 is one of the most well-studied cytokines in terms of its ability to function as a growth factor for T-cells (34). It is also known that pro-inflammatory cytokines such as type I IFNs, IL-12 and IFN-γ all play important roles during the expansion of effector CD8$^+$ T-cells (86). Following the clearance of a pathogen, cytokines such as IL-7 and IL-15 play a central role in maintaining the numbers of antigen-specific memory CD8$^+$ T-cells, such that upon reexposure to the same pathogen, they can respond more rapidly (34, 35, 87). Clearly, cytokines are involved in virtually every developmental step during CD8$^+$ T-cells responses. What is less understood is the role of various cytokines during the recall response of memory CD8$^+$ T-cells, compared to the primary response. It is possible that different cytokines are involved at specific stages, whether it is during the primary or secondary phase of the immune response. As such, in chapter 3 we found that IL-15 plays an critical role during the secondary expansion of memory CD8$^+$ T-cells.

IL-15 TRANSPRESENTATION AUGMENTS MEMORY CD8$^+$ T-CELL RESPONSES

In chapter 3, we addressed the capacity of IL-15 to augment TCR induced activation of CD8$^+$ T-cells. It was demonstrated that transpresented IL-15 is significantly more effective than soluble IL-15 at enhancing TCR induced activation (Fig. 3-1). Previous studies have demonstrated the capacity of soluble IL-15 to induce the activation of CD8$^+$ T-cells and NK-cells (88-98). However, the concentrations of IL-15 used in these studies were extremely non-physiological since soluble IL-15 is virtually undetectable under normal physiological conditions (18-20). Based on our observations, we found that approximately five-fold higher concentrations of soluble IL-15 were required to produce the same effect as when IL-15Rα could transpresent soluble IL-15 (Fig. 3-1). Our studies also demonstrated that memory CD8$^+$ T-cells are more responsive to transpresented IL-15 and more importantly, specific memory subsets displayed differential responsiveness to transpresented IL-15 (Fig. 3-5). Specifically, T_{CM} CD8$^+$ T-cells were found to be highly responsive to transpresented IL-15 alone, or in combination with TCR stimulation with anti-CD3ε or by pMHC. When analyzed *in vivo*, a reduction in T_{CM} CD8$^+$ T-cell proliferative capacity was noted following LCMV infection of IL-15Rα deficient mice (Fig. 3-6) It should be noted that DCs themselves are responsive to IL-15 stimulation and mice lacking IL-15Rα have reduced numbers of circulating DCs that have reduced expression of costimulatory molecules (11, 99). When taken into consideration, it could be argued that the reduced capacity of T_{CM} $CD8^+$ T-cells to proliferate in response to LCMV re-exposure could be the result of reduced numbers of DCs and their lower activation status. However, our *in vitro* data clearly demonstrate that T_{CM} $CD8^+$ T-cells required transpresented IL-15 for optimal recall responses in the complete absence of any other costimulatory ligands (Fig. 3-5*E*). In addition, primary responses against LCMV are normal in IL-15Rα mice suggesting the DCs are capable of expressing sufficient levels of costimulatory molecules required for naïve $CD8^+$ T-cell activation (57).

In chapter 2, we demonstrated that T_{CM} $CD8^+$ T-cells have the highest expression levels of CD122, which may explain their increased responsiveness to transpresented IL-15 (Fig. 2-5*B*). If CD122 were the sole determining factor of responsiveness to IL-15, it would be assumed that T_{EM} $CD8^+$ T-cells with their lower CD122 expression would have reduced responses following TCR stimulation in combination with IL-15 transpresentation. This was found not to be the case, since T_{EM} $CD8^+$ T-cells could proliferate and upregulate grB expression following TCR stimulation alone, which suggests that they have reduced activation requirements compared to T_{CM} $CD8^+$ T-cells (Fig. 3-5*C* and *F*). Taken together, our findings highlight that the activation requirements of T_{CM} and T_{EM} $CD8^+$ T-cells differ significantly in their requirement for IL-15 transpresentation.

T_{CM} VERSUS T_{EM} RESPONSES

Based on the current knowledge of T_{CM} and T_{EM} subpopulations, it is believed that T_{CM} have enhanced proliferative capacity as compared to T_{EM} (43, 100, 101). However, our *in vitro* and *in vivo* results did not demonstrate this as both populations had relatively similar proliferative responses following stimulation (Fig. 3-5 and 3-6). Unfortunately, due to the difficulty in obtaining sufficient numbers of cells for adoptive transfer, the number of LCMV-specific CD8$^+$ T-cells within each memory subpopulation was not determined and may raise potential concerns. For instance, if the T_{EM} CD8$^+$ T-cells had a reduce proliferation capacity, but comprised a large fraction of LCMV-specific CD8$^+$ T-cells, this would result in a significant proportion of proliferating T_{EM} CD8$^+$ T-cells as detected by CFSE dilution. A similar CFSE dilution profile could be obtain if the LCMV-specific T_{CM} CD8$^+$ T-cells had a greater proliferative capacity, but comprised a smaller proportion of the total T_{CM} population. In addition, since T_{CM} CD8$^+$ T-cells proliferate in response to transpresented IL-15 alone, this non-antigen specific proliferating population could also mask the smaller number of proliferating LCMV-specific CD8$^+$ T-cells (Fig. 3-5*D*). Ideally, this experiment should be performed using allelically marked P14 TCR transgenic memory CD8$^+$ T-cells adoptively transferred into congenic wildtype or IL-15Rα deficient mice. In this manner, the specific number of memory CD8$^+$ T-cells bearing a T_{EM} or T_{CM} phenotype could be transferred into each mouse. This approach could also determine if the T_{CM} and T_{EM} dependency on IL-15 transpresentation changes over time. It has been reported that early memory CD8$^+$ T-cell responses are dominated by the T_{EM} subset following respiratory Sendi virus infection (45). This gradually changes so that at later time points, T_{CM} CD8$^+$ T-cell responses dominate the recall response (44, 45). In our experiments, memory CD8$^+$ T-cell populations were isolated at 40 days post-LCMV infection. This may also help explain why T_{EM} proliferative responses were equivalent to the T_{CM} response in our experiments. Based on the understanding that T_{CM} responses dominate at later time points, it would be hypothesized that T_{CM} dependency on IL-15 transpresentation would increase over time (44). Recently, a study delineated multiple memory CD8$^+$ T-cells subsets that develop following Sendai virus infection that do not conform to the T_{CM} and T_{EM} lineages (102). Using the expression of KLRG-1, CXCR3, CD27 and high molecular weight form of CD43, a diverse range of memory CD8$^+$ T-cells subsets with different recall potency were described (102). This highlights the complexity of the memory CD8$^+$ T-cell population and it would be interesting to examine whether these different memory subsets have varying degrees of responsiveness to transpresented IL-15 during recall responses.

COSTIMULATION REQUIREMENTS OF MEMORY CD8$^+$ T-CELLS

Recent studies have demonstrated that DCs are required for the initiation of memory CD8$^+$ T-cell responses (103, 104). This requirement suggests that the reactivation of memory CD8$^+$ T-cells requires either costimulation or cytokines derived from DCs. With respect to naïve CD8$^+$ T-cells activation, the role of costimulation is very well defined, whereas the signals required for memory CD8$^+$ T-cell activation and secondary expansion are only beginning to be understood. We demonstrated in chapter 3 that memory CD8$^+$ T-cell responses are augmented by transpresented IL-15 following TCR stimulation (Fig. 3-4). Furthermore, the T_{CM} CD8$^+$ T-cell population was found to require IL-15 transpresentation for optimal recall responses both *in vitro* and *in vivo* (Fig. 3-5 and 3-6). Due to the requirement of DCs for the reactivation of memory CD8$^+$ T-cells, it is likely that other costimulatory interactions are also involved in initiating secondary responses. Signals provided by the B7/CD28 costimulatory pathway are critical for the induction of primary CD8$^+$ T-cell responses (105, 106). In contrast, memory CD8$^+$ T-cell reactivation is generally accepted to be independent of B7/CD28; however, the support for this notion stems mainly from *in vitro* studies with memory CD4$^+$ T-cells or from the infection of CD28 deficient mice (107-118). Recently however, Borowski *et al.* demonstrated that CD28 costimulation is required *in vivo* for the reactivation of influenza and herpes simplex virus specific memory CD8$^+$ T-cells (119). In the absence of CD28 costimulation, secondary CD8$^+$ T-cell responses and viral clearance were significantly impaired. In addition to B7/CD28 costimulation, there have been reports that members of the TNF family can also influence memory CD8$^+$ T-cell function (120). For instance, 4-1BB is an inducible TNF family receptor expressed by activated CD8$^+$ T-cells that is involved in memory CD8$^+$ T-cell survival and reactivation (110, 111, 121). Interestingly, a link between IL-15 and 4-1BB has been described whereby IL-15 upregulates 4-1BB expression on antigen-specific CD8$^+$ T-cells thereby, enhancing responsiveness to 4-1BBL stimulation (121). This results in the IL-15 independent survival of memory CD8$^+$ T-cells via 4-1BB stimulation. In this manner, the 4-1BB/4-1BBL costimulatory pathway may function more like the survival cytokine IL-7 (122-124). Since IL-15 transpresentation can induce a low level of T_{CM} CD8$^+$ T-cell homeostatic proliferation, IL-15 induced upregulation of 4-1BB could possibly further enhance the survival of the memory CD8$^+$ T-cells following engagement with 4-1BBL. Therefore, it would be interesting to examine the combined effect of transpresented IL-15 and 4-1BBL on the long-term maintenance of memory CD8$^+$ T-cells *in vitro*. Furthermore, since transpresented IL-15, B7, and 4-1BBL all appear to contribute to memory CD8$^+$ T-cell reactivation, it would be very interesting to examine the individual, or combined contribution of these ligands to memory CD8$^+$ T-cell reactivation. These studies could provide potential insight for the design of effective prime-boost vaccination strategies.

PRIME-BOOST VACCINATION

Traditional vaccines have been widely successful in the eradication and control of certain infectious agents such as smallpox and polio (125). Despite these successes, it is apparent that not all pathogens can be controlled by these vaccines since many viruses and tumors can resist the humoral immunity that is typically generated by these approaches (126). Therefore, significant work has been conducted to develop vaccines that elicit cellular immunity to various infectious pathogens and malignancies. Although the development of new vaccine strategies that establish protective CD8$^+$ T-cell memory has proven challenging, heterologous prime-boost schemes may be the answer (125, 127). The principle of prime-boost vaccination involves priming the immune system to a target antigen delivered by one vector and then selectively boosting this immunity by re-administration of antigen in the context of a second and distinct vector. This approach can also refer to the use of different adjuvants during the priming and boosting phases, or the provision of different T-cell costimulatory signals at each stage.

Our work clearly suggests that IL-15 would likely be most effective if provided during the boosting phase to enhance memory CD8$^+$ T-cell recall responses. Other costimulatory molecules such as 4-1BBL have also been suggested to be more effective at the boost stage of a prime-boost vaccination strategy (110, 111). However, since both IL-15 and 4-1BBL are required for the homeostatic proliferation and survival of memory CD8$^+$ T-cells, they may be most effective if provided immediately following initial priming and also during the boosting phase (34, 121).

PRIMARY VERSUS SECONDARY MEMORY CD8$^+$ T-CELLS

The majority of the research conducted in the memory field has dealt with the generation and maintenance of memory CD8$^+$ T-cells that develop following the primary response. In the context of prime-boost vaccination, it is important to consider the differences between primary and secondary memory CD8$^+$ T-cell populations. Secondary memory CD8$^+$ T-cells undergo a protracted contraction phase, which results in their increased numbers following reexposure to the inital pathogen or antigen. Owing to sustained granzyme B expression, secondary memory CD8$^+$ T-cells display enhanced cytolytic ability compared to primary memory CD8$^+$ T-cells on a per cell basis (128, 129). However, as a result of lower CD122 expression levels, secondary memory CD8$^+$ T-cells have reduced responsiveness to IL-15, which results in decreased homeostatic proliferation (128). Therefore, although they are sustained at elevated numbers with enhanced cytotoxic ability, they have a reduced long-term survival capacity. Developmentally, secondary memory CD8$^+$ T-cells have delayed upregulation of T_{CM} characteristics and maintain a T_{EM}-like phenotype (128-132). They are functionally distinct from primary T_{EM} CD8$^+$ T-cells in that they provide significantly better protection against subsequent infections. Because of low CD62L and CCR7 expression levels, secondary memory CD8$^+$ T-cells are excluded from LNs that permits the generation of new primary memory CD8$^+$ T-cells upon reinfection. Taken together, these findings suggest that secondary memory CD8$^+$ T-cells are functionally and phenotypically distinct from primary memory CD8$^+$ T-cells from which they are derived. From results in chapter 3, it is clear that IL-15 transpresentation plays a role in the reactivation of primary memory CD8$^+$ T-cells. Whether the same can be stated for secondary memory CD8$^+$ T-cells is unknown. However, due to their reduced expression of CD122 and diminished responsiveness to IL-15, it could be speculated that they are likely independent of IL-15 with regards to both homeostatic proliferation and reactivation. This suggests that the activation requirements for memory CD8$^+$ T-cells may progressively decrease following repeated stimulation. Alternatively, the activation requirements may shift to alternate costimulatory pathways. Interleukin-15 may therefore play a temporally segregated role in the maintenance and reactivation of primary memory CD8$^+$ T-cells while having minimal effects on naïve and secondary memory CD8$^+$ T-cells (Fig. 5-1).

From these studies, several questions arise when our findings are taken into consideration. In our analysis, we did not examine the phenotype of the resulting secondary effector cells generated following restimulation, nor did we assess their ability to develop into secondary memory CD8$^+$ T-cells. Therefore, do the requirements for homeostatic proliferation change upon secondary expansion? In conditions of chronic infections, CD8$^+$ T-cells require TCR stimulation in addition to homeostatic cytokines for their survival (133). Is this a possibility for secondary memory CD8$^+$ T-cells? The bottom line is that this complex process may serve to benefit the host by generating the most diverse array of antigen specific CD8$^+$ T-cells in situations where frequent pathogen reexposure occurs. Importantly, the ultimate goal of vaccination strategies is to mimic the hosts' natural ability to generate diversity in memory populations.

IMMUNE CORRELATES OF PROTECTION

Following infection or vaccination, the phenotype of CD8$^+$ T-cells that correlate with effective cellular protection must be determined if successful vaccination strategies are to be developed. These include differentiation markers, cytokine expression patterns, and TCR specificity. In recent years, multi-parameter flow cytometry has allowed the simultaneous assessment of each of these characteristics at the single cell level. The careful identification and characterization of phenotypic and functional markers will be critical to the success of vaccine development. This not only applies the identification of new markers, but also the re-examination of long-standing phenotypic markers that have not been adequately assessed since their initial characterization. As such, using a novel mAb that identifies the memory marker Ly-6C, the expression pattern of Ly-6C during acute infection with LCMV was re-examined (Fig. 4-4). It was found that only high expression of Ly-6C^{iMap} on resting CD8$^+$ T-cells correlated with a functional and phenotypic memory CD8$^+$ T-cell (Fig. 4-5 and 4-6). Furthermore, upon analysis of the kinetics of Ly-6C^{iMap} expression, it was found to be upregulated during the acute phase of the immune response, downregulated during the contraction phase, and was maintained by only the antigen-specific memory CD8$^+$ T-cells (Fig. 4-4 and 4-5). Such dynamic expression patterns are not uncommon as it also occurs with other markers such as CD44 that are upregulated immediately following activation and are retained by memory CD8$^+$ T-cells (134). Using multi-parameter flow cytometry to examine the numerical and qualitative aspects of memory CD8$^+$ T-cells will provide valuable insight for the development of successful vaccination strategies.

FUTURE DIRECTIONS

The results described in Chapter 2 and 3 examining the role of IL-15 transpresentation in memory CD8$^+$ T-cell responses was performed independently of the iMap mAb studies in Chapter 4. It would therefore be interesting to look at the responsiveness of iMap sorted $CD8^+$ T-cell populations to transpresented IL-15 alone or in combination with TCR stimulation via pMHC or anti-CD3ε.

Furthermore, the majority of the *in vitro* generated data described in chapters 2 and 3 was obtained using cell-sized microspheres as platforms to examine the individual contribution of IL-15 transpresentation to various immune processes. The versatility and flexibility of this experimental system lends itself to unraveling the activation requirements of $CD8^+$ T-cells. For example, they have been successfully used to identify that inflammatory cytokines such as IL-12 are necessary for the primary expansion of effector $CD8^+$ T-cells (135). In our studies, we have shown that IL-15 transpresentation is required for the secondary expansion of T_{CM} $CD8^+$ T-cells (Fig. 3-5 and 3-6). The next obvious step is to examine the contribution of other costimulatory ligands on memory $CD8^+$ T-cell reactivation. Using microspheres, these questions can be immediately addressed by the co-immobilization of the various ligands in the presence or absence of transpresented IL-15. Understanding the effects of costimulation on the generation of secondary effector and memory $CD8^+$ T-cells would provide potential insight into vaccine formulations for the boost phase of a prime-boost vaccination strategy. Furthermore, the microsphere platform is amendable to examination of human $CD8^+$ T-cell reactivation as many human costimulatory ligands and reagents are becoming readily available.

CONCLUDING REMARKS

From our findings it is clear that transpresented IL-15 plays important roles in the adhesion and costimulation of primary memory $CD8^+$ T-cells, particularly the T_{CM} $CD8^+$ T-cell subset. Developing a greater understanding of how $CD8^+$ T-cells are activated, maintained and restimulated will be critical in the successful development of vaccines and immunotherapeutics. Furthermore, our results suggest that although IL-15 has broad effects on various cell types, within the $CD8^+$ T-cell population, IL-15 may play roles focused on regulating the recruitment, maintenance, and reactivation of primary memory CD8$^+$ T-cells. The hope is that our findings will aid in the rational design and development of immunization protocols for the treatment of human diseases that currently are unresponsive to traditional approaches.

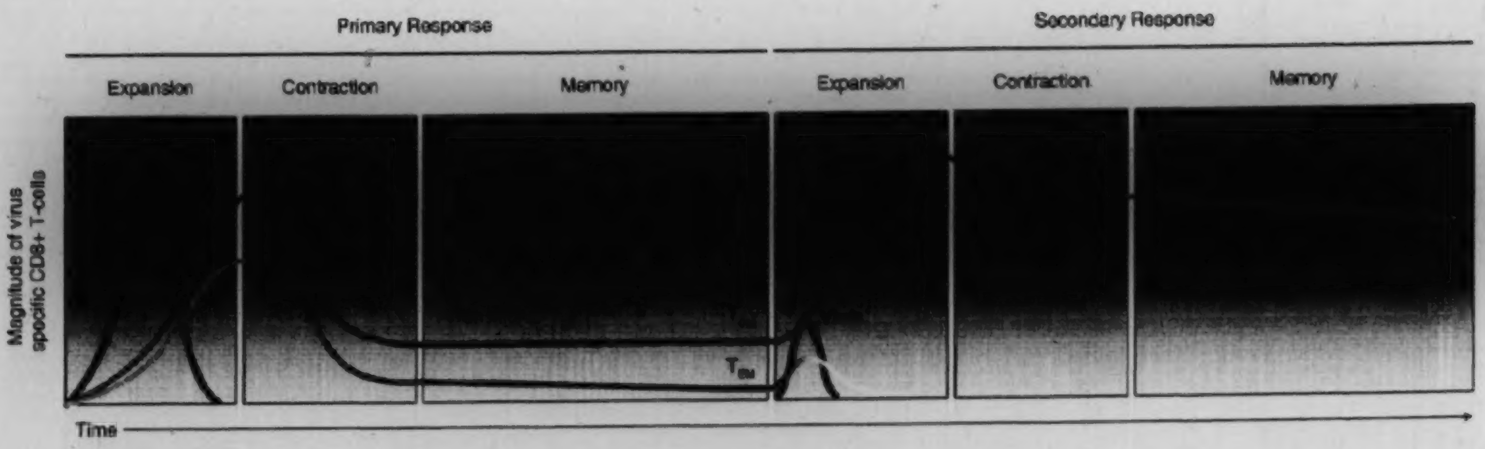
Primary Response
Secondary Response
Expansion
Contraction
Memory
Expansion
Contraction
Memory
Magnitude of virus specific CD8+ T-cells
T_{EM}
Time

Figure 5-1. Interleukin-15 plays a temporally segregated role in the maintenance and reactivation of primary memory CD8$^+$ T-cells. The role of transpresented IL-15 during primary CD8$^+$ T-cell expansion, maintenance, and reactivation is highlighted in red. Transpresented IL-15 is not required for the primary expansion of effector CD8$^+$ T-cells. During the contraction phase, IL-15 can limit the contraction of antigen specific effector CD8$^+$ T-cells, and is absolutely required for the survival and maintenance of T_{CM} CD8$^+$ T-cells during the memory phase. T_{EM} CD8$^+$ T-cells in contrast, are relatively unresponsive to transpresented IL-15 compared to TCM CD8+ T-cells. Following reexposure to the original pathogen, IL-15 transpresentation augments memory CD8$^+$ T-cell reactivation, and is required for optimal recall responses by T_{CM} CD8$^+$ T-cells. Secondary effector CD8$^+$ T-cells generated following reexposure undergo a protracted contraction phase and retain elevated numbers of antigen specific CD8$^+$ T-cells through the secondary memory phase. Owing to reduced expression levels of CD122, the maintenance of secondary memory CD8$^+$ T-cells appears to be IL-15-independent. Therefore, the responsiveness to transpresented IL-15 highlighted in red correlates with elevated expression levels of CD122, and possibly IL-15Rα on the responding CD8$^+$ T-cells.

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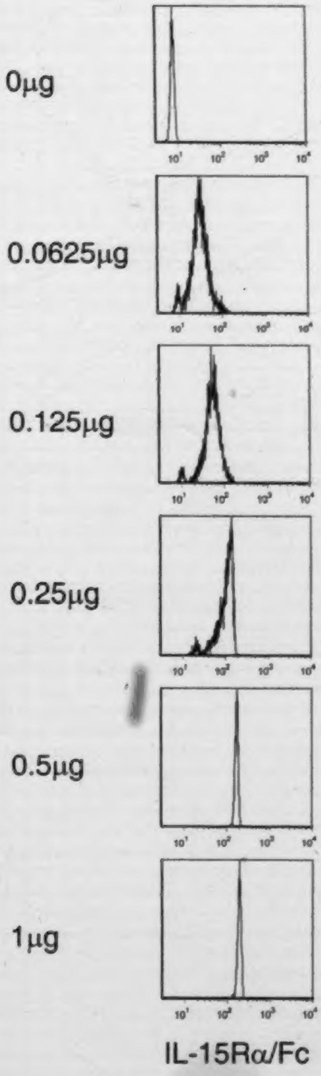

Appendix 1. IL-15Rα/Fc immobilization on paramagnetic M-450 tosylactivated Dynalbeads. Various amounts of recombinant mouse IL-15Rα/Fc was immobilized onto 1×10^7 M-450 tosylactivated Dynalbeads. Following immobilization, microsphere bound IL-15Rα/Fc was detected using a polyclonal goat anti-mouse IL-15Rα/Fc antibody and analyzed by flow cytometry.